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SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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A NOTE ON THE FILTRATION OF THE VIRUS OF HERPETIC ENCEPHALITIS AND OF VACCINIA.

By HUGH K. WARD and FEI-FANG TANG, M.D.

(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston.)

(Received for publication, August 15, 1928.)

I.

Although herpetic encephalitis has long been recognized as due to a filtrable virus, the actual filtration of the virus through the ordinary filters has been attended with a certain amount of difficulty. When the brain of a rabbit dying of herpetic encephalitis is emulsified in saline, the emulsion centrifuged and the supernatant fluid passed through a Berkefeld V filter, our own experience has been that the virus sometimes passes through the filter, and sometimes it does not. This doubt about the infectivity of the filtrate and the consequent necessity of working with the virus mixed with an unfiltered, turbid emulsion of brain tissue have been a hindrance to the study of this virus.

Knowing that the virus of herpetic encephalitis in a saline emulsion of brain tissue, especially when diluted, quickly becomes inactive when exposed to the air, the writers thought that if the filtration of the emulsion could be carried out anaerobically, and the filtrate protected from oxidation, more regularly infective filtrates would be obtained. This hope was not realized, and attention was then turned to the menstruum in which the emulsion of brain tissue was made. Bronfenbrenner (1) had pointed out that if particles carrying bacteriophage were deposited on a Berkefeld filter, the bacteriophage could not be washed through the filter with water, but did pass through if broth was used instead of water. Accordingly, the brain of a rabbit dying of herpetic encephalitis was emulsified in broth (5 gm. of brain in 100 cc. of hormone broth) and the emulsion centrifuged. The supernatant fluid was then filtered anaerobically through a Berkefeld V filter. An ordinary aerobic filtration was carried out at the same time. Both

filtrates were infective, the anaerobic filtrate, kept in an atmosphere of hydrogen, remaining infective for at least 8 days at room temperature. Exactly how long an anaerobic filtrate will remain active, if protected from oxidation, has not been ascertained, as attention was then turned to the conditions necessary for regularly obtaining an active aerobic filtrate from the brain of a rabbit dying of herpetic encephalitis.

Numerous experiments have now been carried out, and in our hands an active filtrate has invariably been obtained with the following technique:

1. The Preparation of the Broth Emulsion of Brain Tissue.—The whole brain of a rabbit which has died of herpetic encephalitis is removed within 24 hours of the rabbit's death. A rabbit showing typical symptoms can of course be killed and the brain removed at once, if it is not convenient to wait for the death of the animal. 5 gm. of the brain are then ground up carefully with sand in a mortar. After grinding for some minutes, hormone broth is added, gradually at first, and then in quantity, until 100 cc. of the broth have been added, the grinding being continued the whole time. This emulsion is then centrifuged for 30 minutes at 2000 revolutions per minute, and the supernatant fluid poured off. A few drops of a fresh culture of *B. prodigiosus* are added to the supernatant fluid, which is then ready for filtration.

2. The Preparation of the Filters.—Berkefeld V filters, after washing through successively with a 1 per cent solution of potassium permanganate, a 5 per cent solution of sodium bisulfite and about a liter of distilled water, are tested for their water flow at a constant negative pressure. The majority of these filters will allow 45 to 60 cc. of distilled water to flow through in 5 minutes at a negative pressure of 10 cm. of mercury. Any filters which differ markedly from these figures are not used. After testing, the filters are autoclaved. The filters are further checked during the actual filtration by the culture of *B. prodigiosus* which is added to the supernatant fluid of the centrifuged emulsion before filtering.

Berkefeld V filters were used throughout in these experiments, but the following filters were tried in one experiment—Berkefeld N, Chamberland L_2, Seitz E.K. and Mandler regular. Of these, only the Mandler filter allowed the virus to pass through. In another experiment, the virus passed through the Berkefeld N filter.

3. The Filtration.—This has been carried out at various negative pressures from 20 to 60 cm. of mercury, and the virus has passed through in all cases. Usually about 30 cc. of the centrifuged broth emulsion are filtered.

4. Testing of the Filtrate.—About 1 cc. of the filtrate is removed sterilely from the filter flask, transferred to a tube of blood broth and incubated for several days to test for the presence of *B. prodigiosus*. If present, growth often does not show before 48 hours, and occasionally even longer than that. We have found quite a number of Berkefeld V filters which allow this organism to pass through. 0.5

cc. of the filtrate is injected intracerebrally into a normal rabbit. If the filtrate contains the virus, the temperature rises on the 3rd or 4th day, and the animal dies from the 4th to the 7th day after injection, after showing the usual symptoms of encephalitis. No systematic experiments have been carried out to ascertain how long and under what conditions the filtrate remains active.

II.

The virus of vaccinia has been filtered by several workers, but it is generally agreed that filtration of this virus is attended with great difficulty. If the same technique which has been described for the filtration of the virus of herpetic encephalitis is applied to the fresh scrapings from the lesions of a vaccinated calf, the virus of vaccinia can be demonstrated in the filtrate by the usual method of scarifying the shaven skin of a normal rabbit. It is clear that there must be a great adsorption of virus by the filter, because the undiluted filtrate gives rise only to discrete papules instead of the confluent eruption caused by the unfiltered emulsion.

From both a practical and theoretical point of view, it is of interest to note that the filtrate is still active after 6 weeks in the ice chest, whether it is in an open tube or sealed with a layer of vaseline or in an open tube diluted with an equal amount of pure glycerol. During this period, no diminution in the activity of the filtrate could be detected.

It has been found that if the scrapings are kept on ice for 2 or 3 days before emulsifying in broth and filtering, the virus can no longer be demonstrated in the filtrate, although the unfiltered, centrifuged emulsion is still very active. Further, if the fresh scrapings are emulsified in 50 per cent glycerol broth (in a proportion of one part of scrapings to four parts of 50 per cent glycerol broth) and this emulsion kept on ice for some days before adding fifteen additional parts of broth (*i.e.* a final 5 per cent emulsion of the scrapings), the filtrate is again negative.

While these experiments were in progress, Mills, Shibley and Dochez (2) in America, and Garrod (3) in England have published papers on a method of obtaining filter-passing organisms from the nasopharynx, using broth instead of saline or phosphate buffer solution to obtain the nasal washings for filtration. The filtrates of the saline washings were almost uniformly negative, while organisms were regularly found in the filtrates of the broth washings.

DISCUSSION.

This note is published in the hope that it will help in the study of filtrable viruses in general. The difficulty in filtering certain of the filtrable viruses has been sometimes ascribed to the intracellular location of the virus, *e.g.* vaccinia, the filter removing the cells and therefore the infecting agent. It is clear from these experiments that the virus of vaccinia, even if it is ordinarily intracellular, can easily be separated from the cells without destroying its infectivity. Further, if careful centrifugation experiments were done on the filtrate, as Gordon (4) has done on the emulsion, it might be possible to throw some more light on the probable size of the infecting agent. Again, the question of whether or not there are complement-fixing and precipitating antibodies in neutralizing serum might be reinvestigated, with an antigen which is free of organisms and cells. From the practical point of view, the possibility is opened up of preparing vaccine for human vaccination quickly and easily, removing the contaminating organisms completely by filtration, instead of incompletely removing them by the long drawn out process of partial sterilization.

We have not been able so far to throw very much light on the size of the herpes virus by these filtration experiments. The fact that the virus invariably passed through a Berkefeld V filter and in the first experiment was stopped by a Berkefeld N filter was encouraging, but in a second experiment the virus passed through the N filter. Perhaps a second filtration of the clear Berkefeld V filtrate through graded filters might give a truer indication of the size of the virus.

SUMMARY.

The virus of herpetic encephalitis and the virus of vaccinia can be demonstrated in the filtrate, if a broth emulsion of fresh tissue containing the virus, is passed through a Berkefeld V filter.

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A CHEMICAL AND PATHOLOGIC STUDY OF THE EFFECTS OF COPPER ON THE LIVER.

By FREDERICK B. FLINN, Ph.D., and WILLIAM C. VonGLAHN, M.D.

(*From the Departments of Industrial Hygiene and Pathology, College of Physicians and Surgeons, Columbia University, New York.*)

Plate 1.

(Received for publication, August 11, 1928.)

For many years the question of the toxic effects of copper ingested over long periods has been the subject of investigation. Many of the original reports of the earlier investigators, unfortunately, could not be obtained by us and it was only from citations from the later authors that any idea of their conclusions could be had. The results were often found to be conflicting.

Filehne (1895) (1) testing the effect of cupratin, made by heating sodium albuminate with copper sulfate, found that large doses given to cats and dogs produced vomiting, but no pathologic alterations in the liver could be discovered. Neither did an anemia develop. About 98 per cent of the copper was eliminated in the feces. His conclusion was that food containing copper combined with albumin was harmless. In later experiments using copper stearate, he (2) found that pigment was deposited in the portal areas of the rabbit's liver while the parenchymal cells contained fat globules. Metallic copper and copper tartrate did not produce any ill effects.

Brandl (3) investigating the subject a little later reached a similar conclusion.

In 1918 Huber (4) studied the effect of the copper salts of amino acids. These compounds were given to guinea pigs by various methods—subcutaneously, intramuscularly and by mouth. In the chronically poisoned animals the results were entirely negative.

Mallory, Parker and Nye (5) studying the relation between experimental pigment cirrhosis and hemachromatosis found pigment deposited in the liver of the rabbit, rat, guinea pig and monkey in chronic copper poisoning. Copper acetate, copper chloride and cuprohemol were given to the animals. The pigment was deposited more rapidly in the liver of the rabbit and rat than in the guinea pig. In the rabbit the deposition took place in the liver, especially in those cells near the portal areas in a short time, and in the endothelial cells lining the sinusoids. Many of the liver cells became necrotic and were invaded by polymorphonuclear

leucocytes. Pigment granules were taken up by the endothelial phagocytes which then collected in large numbers in the lymph vessels and between the fibroblasts in the portal areas. Occasionally pigment was found also in the fibroblasts but never caused necrosis of these cells. The pigment deposited in the liver did not give the reaction for iron and was identified as hemofuscin. No evidence of blood destruction was found. In a monkey, dying after copper poisoning of 5 months duration, pigment was present in the liver cells. About half of the pigment gave the reaction for iron. Another monkey was still alive at the end of 15 months. It had been given smaller doses of copper.

The first increase in connective tissue occurred near the portal vessels. Here the liver cells containing more pigment than elsewhere underwent necrosis, allowing the stroma to coalesce forming foci of sclerosis. The earliest definite cirrhosis in the rabbit occurred at the end of 5½ months. Stasis of bile was found. A second case came at the end of 6½ months. This was accompanied by jaundice. A third rabbit died from cirrhosis after 11½ months. Individual variations in the response to the poisonous effects of copper were found among the rabbits, even those of the same litter.

One of their conclusions was that "chronic poisoning with the salts of copper produced in the livers of rabbits in 6 months to a year a series of changes comparable in many ways with those found in the liver in a chronic disease in man known as hemachromatosis."

In a later publication Mallory (6) states that sheep are extremely sensitive to copper poisoning and are killed by doses no larger than those effective with rabbits. He also found delicate hemofuscin granules in the liver cells in the periportal region of a rabbit dying within 36 hours after two intravenous injections of copper powder. The animal was first given 50 mg. in a gelatin suspension and 24 hours later 100 mg. When copper is injected intratracheally into a rabbit, necrosis of the lung is produced with an acute inflammatory reaction. After 10 days the liver cells contained considerable hemofuscin; some was present also in the endothelial cells of the sinusoids and to a slight extent in the fibroblasts.

Drummond (7) investigating the deleterious effects of vegetables artificially colored with copper obtained only negative results. His experiments were carried out on rats and were continued for 3 months.

The most recent studies of the relation between pigment cirrhosis and copper poisoning are those of Hall and Butt (8). Rabbits, sheep and rats were given copper acetate in various ways. Their results confirmed the findings of Mallory and his coworkers. They believe a direct relation exists between the amount of copper contained in the liver and the quantity of pigment deposited, and that the pigment—hemofuscin—contains iron in masked form.

The Copper Content of Human and Animal Livers.

During the course of the experiments recorded below, numerous human and animal livers were analyzed to determine the normal cop-

per content. The analyses were carried out by the following method:

The material was carefully ashed at low temperature in an electric oven and completely dissolved in concentrated nitric acid, then evaporated down to white

TABLE I.

The Copper Content of Human Liver.

Autopsy number	Age	Sex	Copper per kg.	Anatomic diagnosis
	yrs.		*mg.*	
9828	42	M.	7.14	General arteriosclerosis; occlusion of coronary artery; infarct of heart
9838	70	M.	12.42	Fibrosarcoma of pelvis
9845	37	F.	3.86	Chronic nephritis
9847	53	M.	5.04	Syphilitic aortitis
9849	73	M.	6.36	General arteriosclerosis; aneurysm of aorta
9851	40	M.	4.62	Syphilitic aortitis
	mos.			
9852	9	M.	3.92	Confluent lobular pneumonia
	yrs.			
9853	52	M.	2.30	Lobar pneumonia; syphilitic aortitis
9856	34	F.	4.66	Tuberculosis of lymph glands, appendix and peritoneum
9857	41	M.	3.00	Subacute bacterial endocarditis
9859	27	F.	2.40	Chronic myelocytic leucemia
9858	25	F.	4.05	Radium poisoning; necrosis of jaw
9861	8	F.	4.76	Typhoid fever
9863	44	M.	3.00	Chronic nephritis
9865	60	F.	4.50	Chronic cholecystitis; cholelithiasis
9862	30	F.	4.02	Lobar pneumonia
9868	51	M.	7.16	Carcinoma of transverse colon
9866	59	M.	4.32	Acute cholecystitis; cholelithiasis; hemorrhagic pancreatitis
9869	55	F.	3.40	Carcinoma of stomach
9872	25	F.	5.36	Acute myeloblastic leucemia
*	?	M.	3.74	?
**	41	M.	5.34	Strychnine poisoning

* Worker in brass foundry.

** Copper miner.

fumes after the addition of a few cc. of concentrated sulfuric acid. The residue was dissolved in boiling water and the copper plated out on a platinum cone by means of an electric current. When no more copper was deposited the cone was

removed and the copper dissolved off in a small amount of concentrated nitric acid. The cone was then returned to the original solution and the electroplating continued for another 24 hours. The solution containing the copper was evaporated down to about 0.2 cc. and made alkaline with ammonia. From the depth of the blue color the amount of copper was estimated. This result was checked by the thiosulfate method, 0.005 normal solution of thiosulfate being used and the standard procedure for the determination of the amount of copper by this method being followed.

Human Livers.—Twenty specimens of liver were obtained from routine necropsies and were selected from those which were normal. In addition, two other specimens were examined from workers in

TABLE II.

Copper Content of the Liver of Various Animals.

	Copper content per 100 gm. of liver		Copper content per 100 gm. of liver
	mg.		*mg.*
Goat	15.6	Grey rat	3.3
Sheep	9.8	White rat	3.4
Cow	2.60	Gila monster	1.75
Rabbit	1.12	Guinea pig	3.1
Cat	9.20	Turtle	5.26
Cryptobranch	1.8		

copper industries, one in a copper mine for more than 14 years, the other in a brass foundry for several years.

The copper content of the livers varied from 2.30 to 12.42 mg. per kilo, and it is interesting that the content of the livers from the workers in the copper industries was close to the minimum (Table I).

Animal Livers.—The copper content of the liver of various animals is shown in Table II.

Experimental Effects of Copper on Animals.

The ordinary laboratory animals were used for the various experiments.

For reasons which will become apparent later, it was desirable to control the diets. In general the animals were maintained upon diets recognized as adequate, but in certain experiments special diets were used. The copper and its salts and the sodium acetate given to the animals were chemically pure products.

The specimens for histologic examination were fixed in 10 per cent formalin and in Zenker's fluid without the addition of acetic acid. The routine stains were hematoxylin and eosin; duplicate sections were prepared by the method of Mallory, Parker and Nye for demonstrating hemosiderin and hemofuscin.

Guinea Pigs.—Animals weighing approximately 450 gm. were used. A careful record was kept of the weight curve and a blood count was made at regular intervals. The results of these counts will be reported by Flinn in another publication.

The diet consisted of hay and oats daily, supplemented with carrots and cabbage on alternate days. The animals gained in weight steadily on this diet. It was found that guinea pigs ate sparingly of the carrots and, when given carrots exclusively, refused to eat.

Ten guinea pigs were chosen for the experiment. For the first 5 months they were given, daily, copper chloride equivalent to 0.20 mg. of copper. This was mixed with 120 mg. of lactose and packed into a gelatin capsule. To insure that the animal received the specified dose, the capsule was placed in the pharynx and the jaws tightly closed until it had been swallowed.

At the end of 5 months, the copper chloride was discontinued and 1 mg. of metallic copper substituted. The copper was ground so finely that most of it would pass through silk cloth. It had been previously determined that copper so finely ground was soluble in plasma, as much as 7.4 mg. being readily dissolved in 100 cc. of plasma at the end of 24 hours, without agitating the mixture. The copper powder was mixed with lactose and given to the guinea pigs in capsules. This dosage was continued for 13 months and the animals then killed. The total length of time of copper ingestion was 18 months.

The results with guinea pigs were entirely negative. Pigment had not been deposited in the liver nor any cirrhosis produced. This finding is in accord with that of Huber. Mallory, Parker and Nye found guinea pigs more resistant to the effects of copper than are rabbits. In a later publication Mallory (9) states that guinea pigs seem to be almost or entirely immune to chronic copper poisoning.

White Rats.—Eighteen rats averaging 75 gm. each were selected for the first experiment. They were placed upon a diet of mixed grains—wheat, oats and corn—and, in addition, were given powdered milk, with raw vegetables from time to time. It had been found before beginning the experiment that rats of the size used drink an average of 10 cc. of water daily during the autumn, spring and winter months. In summer their water consumption rises to 15 cc. per day. The copper was given as the chloride dissolved in the drinking water. To reduce loss from evaporation and spilling, the water was placed in an inverted burette with a very small opening at the bottom. They readily drank from this. Rats. Nos. 16 to 30, were given water containing the equivalent of 1 mg. of copper in each 10 cc. and Nos. 35 to 43, 2 mg. of copper in each 10 cc. The remaining rats,

Nos. 46 to 78, were allowed water containing 1 mg. of copper per 10 cc. At the end of 6 months the copper content was increased to 2 mg. The number of days the animals were allowed to live is shown in Table III.

The amount of copper being excreted in urine and feces was determined for some of the animals. During these tests the rats were kept in separate cages. The individual average daily copper excretion of twelve rats over a period of 7 weeks was found to be 0.97 mg. in feces and 0.016 mg. in urine. The livers of some were also analyzed for copper. The results are given in Table III.

A second experiment was undertaken with seven rats from the same litter. They were given water containing the equivalent of 2 mg. of copper, as the chloride, in each 10 cc. The experiment was begun as soon as the animals could drink water and was continued for 547 days. At the end of the experiment they weighed from 112 to 130 gm. each.

TABLE III.

Rats.

Number	Number of days	Copper in the whole liver	Number	Number of days	Copper in the whole liver
		mg.			*mg.*
16	147	0.1	46	192	
21	154		54	327	0.26
25	160		61	351	0.8
26	161		63	365	
28	199		73	426	
30	240		76	438	0.29
			78	469	0.26
35	120	0.6			
36	120				
39	120				
40	120	0.34			
43	120				

Only negative results were obtained with white rats. No pigment had been deposited in the livers, neither was there any cirrhosis. These findings coincide with those of Drummond but are not in accord with those of Mallory and his coworkers, nor of Hall and Butt.

Rabbits.—Thirty-four full grown Belgian hares, weighing about 2 kilos each, were divided into groups as enumerated below. The length of time the animals were given copper or one of its compounds, and the copper content of many of the livers, are shown in the appropriate table. They were fed upon a diet consisting of hay and oats in unrestricted quantity, with carrots and cabbage on alternate days.

TABLE IV.

Rabbits. Copper Chloride, 100 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
5	130	1.30
6	207	2.85
7	207	2.80
8	207	1.74
9	207	1.70

TABLE V.

Rabbits. Metallic Copper, 20 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
2-88	257	10.24
2-95	162	10.30
2-91	269	2.10
2-85	300	4.20

TABLE VI.

Rabbits. Metallic Copper, 134 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
13	30	No assay
17	28	No assay
70	15	20.74
19	35	No assay
71	103	36.4
18	37	84.9

TABLE VII.

Rabbits. Metallic Copper, 50 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
2-42	19	5.50
2-59	142	4.05
2-94	103	7.42

TABLE VIII.

Rabbits. Metallic Copper, 320 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
14	44	38.90
11	7	41.40
12	14	40.10
16	40	41.50

TABLE IX.

Rabbits. Copper Acetate, 100 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
10	248	1.68
1-70	24	4.64
4-73	36	0.60
2-25	45	1.56
2-03	283	6.75

TABLE X.

Rabbits. Copper Acetate, 100 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
1-02	19	9.20
1-05	69	15.82
1-34	36	3.78
1-99	69	3.48

TABLE XI.

Rabbits. Copper Tartrate, 100 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
2-54	94	2.97
2-51	177	3.53
2-52	177	4.32

This will hereafter be referred to as the standard diet. Metallic copper, in the finely ground form, and copper chloride were administered in gelatin capsules. Copper acetate and copper tartrate were mixed with a little chopped cabbage and the animals watched until the cabbage had been eaten. The amount of copper or its compounds indicated below is the daily dose.

Group 1. Five rabbits. Copper chloride, 100 mg. Standard diet. Table IV.

Group 2. Four rabbits. Metallic copper, 20 mg. Standard diet. Table V.

Group 3. Six rabbits. Metallic copper, 134 mg. Standard diet. Table VI.

Group 4. Three rabbits. Metallic copper, 50 mg. Standard diet. Table VII.

Group 5. Four rabbits. Metallic copper, 320 mg. Standard diet. Table VIII.

Group 6. Five rabbits. Copper acetate, 100 mg. Standard diet. Table IX.

Group 7. Four rabbits. Copper acetate, 100 mg. Standard diet. Table X. Food was withheld from these animals for 4 hours, then the copper acetate was given in chopped cabbage. It was hoped by this procedure to increase any effects of the copper. All food was removed from the cages during the night.

Group 8. Three rabbits. Copper tartrate, 100 mg. Standard diet. Table XI.

Results.—The results on these various doses of copper or its compounds were uniform for all groups of rabbits. The histologic alterations in the livers of the animals were so alike that a detailed description of each animal or group is unnecessary.

The liver was always the site of pigment deposits (Fig. 1). The pigment occurred in fine refractile particles having a yellow or yellowish brown color. Though most abundant in the cells about the portal areas it was without exception found in all of the parenchymal cells of

the lobule. There was, however, no fixed relation between the amount of pigment and the length of time the copper was administered. Thus Rabbit 2-03 which had received 100 mg. of copper acetate daily for 283 days had less pigment than did Rabbit 2-25 of the same group treated for only 45 days. Similar variations in the amount of pigment deposited were encountered in the various groups and no difference could be detected as regards the pigment deposition and the form in which the copper had been given. No correlation could be found between the amount of copper contained in the liver and the quantity of pigment deposited.

Pigment was also present in the portal areas of seven of the animals and was usually held within phagocytes. Occasionally connective tissue cells in the same region contained pigment. In two of these seven animals a little of the pigment had been engulfed by the endothelial cells of the sinusoids.

The problem of deciding whether the connective tissue of the portal areas was increased beyond normal limits presented difficulties. Variations in the amount of fibrous tissue in this region were noted in the same section and in the various animals receiving the same treatment. In some, the connective tissue was loosely arranged, in others, dense. In one animal, No. 1-70, Group 6, a single portal area was found with a few new bile ducts. This animal had only had twenty-four doses of copper acetate. The pigment in its liver was in moderate amount and none could be found in the portal areas or endothelial cells. It does not seem likely that the copper salt could have produced this effect in so short a time.

In no single animal was the amount of connective tissue sufficiently increased or widespread to justify a diagnosis of cirrhosis. Neither did any animal become jaundiced, nor was any bile stasis made out on histologic examination of the livers.

Large focal necroses were encountered in various liver sections. These could not be related to the ingestion of copper and are interpreted as due to intercurrent infections. Necrosis of individual liver cells could not be found.

Staining Reactions of the Pigment.—In hematoxylin-eosin preparations the pigment remained unstained. No staining of the pigment in frozen sections of formalin-fixed tissue could be discerned with osmic

acid, Scharlach R or Sudan III. Nile-blue sulfate, saturated aqueous solution, colored the pigment greenish blue to deep blue. Fat was not demonstrated in association with the pigment by any of these stains. With fuchsin, by the method described by Mallory, Parker and Nye, to demonstrate hemosiderin and hemofuscin, the pigment invariably stained red or red with a faintly yellow tint. As a control for this method the spleen and liver were embedded together and the sections of the two organs were thus mounted on the same slide. The spleen always contained hemosiderin and this served to check the proper employment of the method.

TABLE XII.

Rabbits. Sodium Acetate, 100 Mg. Daily.

Number	Number of days	Copper content per 100 gm. of liver
		mg.
1-98	180	1.0
2-20	180	0.34
1-41	180	0.34
2-75	180	0.34
2-28	180	0.34
2-92	180	0.68
1-97	180	0.78
2-69	180	0.54
2-36	151	No assay
2-72	151	No assay

By referring to the various tables it will be noted that there is no constant relation between the amount of copper contained in the liver and the type of copper given. Variations in the quantity of copper in the organ are found within the same group as well as in the different groups. The largest amounts of copper were held in the livers of those receiving metallic copper and even in these groups there is a wide variation between the amount ingested and the liver content.

Effect of Sodium Acetate.

It seemed of interest to determine whether the acid radicles in the copper salts were of importance in influencing the deposition of the pigment. With this in view ten rabbits were given 100 mg. of sodium

acetate, daily, in chopped cabbage. The animals were fed upon the standard diet (Table XII). At the end of 5 months two of the rabbits were killed; the remaining eight were allowed to live a month longer.

It was found on histologic examination of the livers that pigment had been deposited in the liver cells in amounts comparable with that in the animals receiving copper (Fig. 2). In one, a few of the endothelial cells of the sinusoids contained pigment, and in four, pigment was present in phagocytes in the portal areas. The pigment conformed in staining reactions with that in the copper-fed animals. It is needless to give a detailed description of the amount of connective tissue in the portal areas. The findings were similar to those in the animals receiving copper. The amount of copper contained in the livers of some of these rabbits is shown in Table XII and these results may be taken as the amount which occurs in that organ when the animal is kept on the standard diet.

This experiment made it obvious that copper was not the agent causing the pigment deposition in the various groups of rabbits to which it had been given.

Effects of Diet.

The result obtained when sodium acetate was given to rabbits as outlined above showed the necessity of seeking other factors causing the pigment deposits, and also raised the question of possible occurrence of pigment in the liver cells before beginning any experiment. For a source of pigment which might be deposited in the liver cells without the intervention of the metal, one naturally turned to the diet. As all of the animals in the previous experiments had been fed the standard diet consisting of hay, oats, carrots and cabbage, carrots were selected as the most probable source of the pigment.

To determine the amount of pigment which might be present in the liver before beginning some of the experiments, pieces of the organ were removed under ether anesthesia and studied. The same staining methods were employed as outlined previously.

The Occurrence of Pigment in the Livers of Normal Rabbits.

Eighteen rabbits were operated upon and specimens of liver removed for histologic examination. These animals were then used for further experiments.

In each of the eighteen, pigment was found in the hepatic cells. A few granules of pigment were present in seven of the specimens and in eight the liver cells contained pigment in small quantities. In these fifteen rabbits the pigment was confined to the cells near the portal areas, and it is evidently in the cells of this zone that the deposition first takes place. In three animals the pigment had been deposited in moderate amount and was more widely distributed throughout the lobule. This pigment stained as did the pigment in the livers of the copper-fed animals. In three of the control animals a few portal areas had an excess of cells with fusiform nuclei, a change similar to that seen in some of the animals after the ingestion of copper compounds and in the group given sodium acetate.

Effect of Diet Consisting of Carrots Only.

Ten full grown rabbits were placed upon a diet consisting of unrestricted quantities of carrots only. From none of these had pieces of liver been removed before the diet was begun. The animals were killed at varying intervals and pigment demonstrated in each liver.

At the end of a week the pigment quantity did not exceed that seen in some of the control rabbits. After 2 weeks the pigment was greatly increased and, in one case, was in excessive amounts, occurring also in some of the endothelial cells of the sinusoids as well as in phagocytes in the portal areas. In another animal kept on the diet for 2 weeks the hepatic cells contained large amounts of the pigment. In the liver of the rabbit examined at the end of 3 weeks, the hepatic cells contained pigment in large amounts but none could be discovered in either the sinusoidal endothelial cells or the portal areas. Of the three animals examined after 4 weeks, one had an abundance of pigment in the liver cells only; in the other two pigment was present also in cells in many of the portal areas, while in one of these two, some of the sinusoidal endothelial cells contained pigment (Fig. 3). After 7 weeks the pigment was not increased in the liver as compared with that found at 4 weeks; some was present in cells in the portal areas. In neither of the two remaining animals killed after being on carrots for 15 and 19 weeks respectively, was the pigment as abundant as in those animals examined at the end of 4 weeks. However, it greatly exceeded in amount that which existed in the livers of any of the control animals. Focal necroses were found in three of the livers; these are not interpreted as a result of the accumulation of pigment in the cells.

With three exceptions, the amount of pigment found in the liver was roughly proportional to the length of time the diet was continued.

These three exceptions were the animals which were continued longest on the diet. No explanation could be discovered for this exceptional reaction. The pigment conformed in its staining reactions with that in the livers of the rabbits given copper or its compounds or sodium acetate. Identical variations in the quantity of connective tissue in the portal areas were found in this group as existed in the rabbits given copper or its salts. A detailed description would be but a repetition of what has been said in that connection.

Each rabbit consumed an average of 360 gm. of carrots daily. The copper content of the undried vegetable was found to be 1.30 mg. per kilo. The individual animal would have ingested not more than 0.50 mg. of copper daily and it is impossible to attribute the rapid deposition of pigment to so small amount of the metal.

Further experiments will be undertaken to determine how long the pigment will remain in the liver and whether such pigment deposits may eventually lead to cirrhosis.

The Effect of Copper with Carrot Diet.

Another attempt was made to demonstrate the relation between copper and hepatic pigment deposition. In the experiment just outlined, a diet of carrots was found to produce pigmentation of the liver. If copper were an etiological factor in pigment accumulation, then a combination of copper with carrot diet should produce even greater deposits.

Accordingly three animals were fed upon carrots and given 100 mg. of copper acetate daily. Specimens of liver were removed before the experiment was begun. Each contained pigment in small amounts in the cells near the portal areas. These animals are included among the eighteen normal rabbits. At the end of 42 days, two of the rabbits were killed. The pigment in the liver cells was definitely increased but no difference could be detected in the amount of connective tissue in the portal areas.

The third animal was allowed to live for 88 days. Pigment was more abundant than at the beginning of the experiment. No increase in the connective tissue of the portal areas had taken place. In none of these animals did any of the endothelial cells of the sinusoids contain pigment, nor was any found in the portal areas. The staining reactions of this pigment were identical in every respect with the pigment in the other animals.

The pigmentation found in these animals did not equal that of one of the rabbits fed on carrots for 14 days, nor was it at all comparable to the deposition which occurred on carrot diet for 28 days. The only explanation offered for this is a probable loss of appetite and a lessened ingestion of carrots.

Results on a Diet of Turnips.

Ten full grown rabbits were operated upon and pieces of liver removed for study. These are included in the eighteen described above. Each of these ten had pigment in the liver cells, eight of them in small amounts and two in moderate quantities.

Five of the animals were placed upon a diet of peeled *yellow turnips* only. Two died of diarrhea after 2 days and were discarded. The other three animals also had diarrhea.

One animal died at the end of 9 days. The pigment in the liver cells was slightly but definitely increased in amount. Another died after 25 days. As compared with that present in the liver removed before the diet was begun, the pigment was increased but not markedly so. A greater increase in the amount of pigment was found in the liver of the animal which died on the 27th day of the diet. In none of these animals was pigment present in the sinusoidal endothelial cells, nor in the portal areas.

The other five rabbits were given a diet of peeled *white turnips* exclusively. Pigment was found to be present in the livers of each of these five by removing a specimen before beginning the diet. In four, the pigment was in small amounts; in the other, in moderate quantity.

Four of the animals had diarrhea. One died at the end of 9 days. The pigment in the liver was not increased. Another died after 13 days; the pigment in the liver cells had increased slightly. In the animal dying on the 17th day, the amount of pigment in the liver was definitely greater than in the control section. In the liver of the animal which died on the 31st day, the pigment had increased in amount. A few cells in one portal area contained some pigment. At the end of 7 weeks, the remaining rabbit of the series was killed. No change in the quantity of pigment could be detected.

As determined by staining reactions, this pigment was of the same nature as that found in the livers of the rabbits fed on carrots alone, and in those given copper or sodium acetate with the standard diet.

The increase in pigment in the livers was not striking on the diets of white or yellow turnips; it did not exceed that found in the liver of the rabbit fed on carrots alone for 1 week, and was not nearly so abundant as in the livers of rabbits fed on carrots for 2 weeks or longer.

DISCUSSION.

From the foregoing experiments it is apparent that copper is not the cause of the pigment deposits in the liver of the rabbit, since the same change is produced when sodium acetate is given with the standard diet. Furthermore, when rabbits are fed on a diet of carrots exclusively, the deposition of pigment occurs with greater rapidity than with copper or its compounds and the standard diet. The changes resulting in the livers of the carrot-fed animals are identical in every way with those seen following large doses of copper and in animals given comparable doses of sodium acetate. On a diet of peeled turnips, pigment is deposited in the liver of the rabbit though much more slowly than with carrots.

In none of the rabbits given copper could a definite cirrhosis be proven. It is true that in some of the portal areas there appeared to be a slight increase in the number of connective tissue cells. This also was readily made out in the sodium acetate animals and in those on a diet of carrots. The increase in the connective tissue was so slight that one would hesitate to make a diagnosis of cirrhosis or to conclude that this change had been experimentally produced, especially in view of the fact that an increase in the connective tissue in the liver occurs so frequently in supposedly normal rabbits (10). Even if it might, in the opinion of some, be considered a cirrhosis, its occurrence in the livers of the animal given sodium acetate as well as in those on a diet of carrots, would exclude copper as the etiological factor. As to the manner in which the pigment reached the portal areas, nothing definite could be determined. In this situation the pigment was more often in phagocytic cells, and only occasionally in the connective tissue elements. Necrosis of individual liver cells was carefully sought for, but could not be found.

Regarding the nature of the pigment nothing definite can be said at the present time. Concerning its source, the evidence indicates that it is of exogenous origin, for it is found in such great abundance in the rabbits fed upon carrots only, and in lesser amounts in those on a diet of turnips.

CONCLUSIONS.

From the above studies the following conclusions have been reached:

1. That copper or its compounds used does not cause the deposition

of pigment in the livers of rabbits, guinea pigs or rats. Neither does it produce a cirrhosis in these animals.

2. That spontaneous deposition of pigment occurs frequently in the livers of normal rabbits on the usual laboratory diet.

3. That the feeding of a diet of carrots exclusively will produce pigment deposition in the livers of rabbits, in every way identical with that ascribed to copper.

4. That the pigment deposited in the livers of rabbits is probably of exogenous origin.

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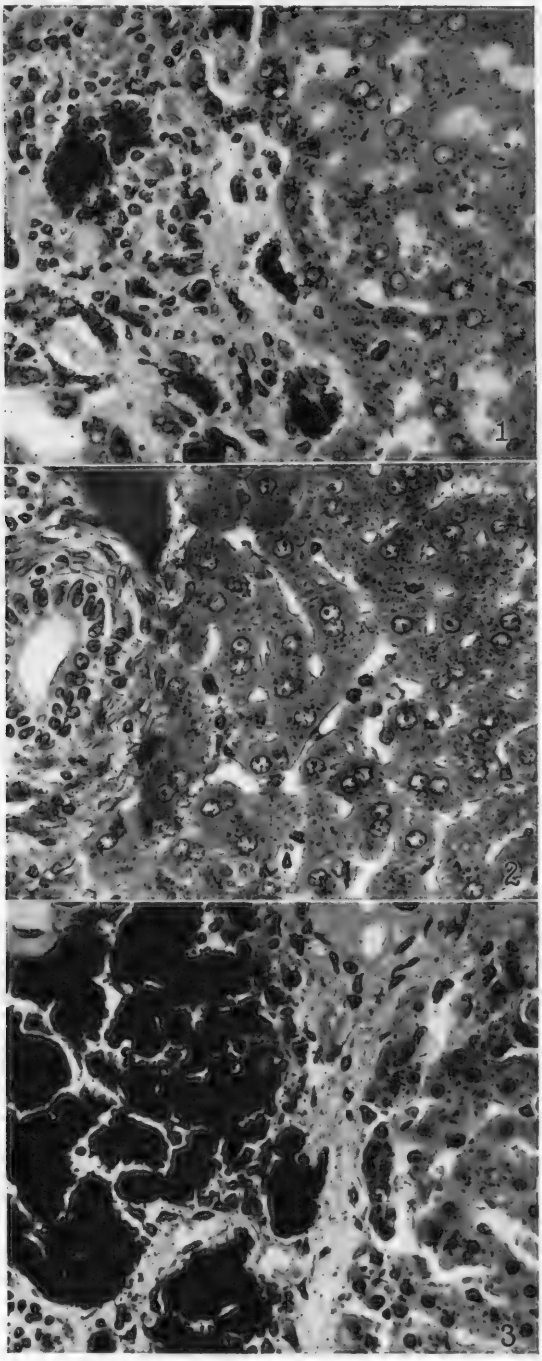
EXPLANATION OF PLATE 1.

FIG. 1. Rabbit 2-85. Metallic copper, 20 mg. daily for 300 days. Pigment in liver cells and within phagocytes in portal area. (Fuchsin stain. × about 415.)

FIG. 2. Rabbit 2-36. Sodium acetate, 100 mg. daily for 151 days. Pigment in liver cells. (Fuchsin stain. × about 415.)

FIG. 3. Rabbit 3-56. Diet of carrots only for 28 days. Pigment in liver cells and within phagocytes in portal area. (Fuchsin stain. × about 415.)



(Flinn and VonGlahn: Effects of copper on liver.)

STUDIES IN ASTHMA.

VII. The Pulmonary Circulation in the Guinea Pig during Anaphylactic Shock.*

By STEPHEN WENT,† M.D., and CECIL K. DRINKER, M.D.

(*From the Department of Physiology, Harvard School of Public Health, Boston.*)

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In 1924, Drinker and Bronfenbrenner (1) measured the pressure in the pulmonary artery of the rabbit, the cat, the dog and the monkey during and for a brief period after the intravenous injection of small doses of sheep serum. The animals in question had received antecedent doses of this protein. It was found that in rabbits, anaphylactic shock is characterized by a notable degree of pulmonary vasoconstriction, great enough in some instances to cause the death of the animal. The same reaction but in lesser degree was observed in cats. Shock in dogs was without expression in the pulmonary circuit and as has been the invariable experience; no gross phenomena of any sort followed the second injection in monkeys.

At the time these experiments were made, measurement of the pulmonary arterial pressure in the guinea pig under good physiological conditions had not been accomplished. During the past spring the authors (2) have devised a method for making this measurement, which is so simple and reliable as to induce them to carry out the missing experiments upon this animal.

Auer and Lewis (3) in their paper on the immediate reaction of anaphylaxis in the guinea pig measured the systemic blood pressure during "shock" as have also Anderson and Schultz (4) and Loewit (5). All these authors have shown a rise in this pressure, coincident with the development of asphyxia. The absence of pronounced changes in the action of the heart constituted presumptive evidence that

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† Fellow of the Division of Medical Education of the Rockefeller Foundation.

nothing notable occurred in the pulmonary blood circuit during shock, but without explicit experiment this could not be considered certain.

Technique.

Anesthesia.—All of the guinea pigs were anesthetized by intraperitoneal injections of 5 per cent sodium-barbital. It seems certain that this anesthetic, in common with others, somewhat reduces the acuity of anaphylactic shock but does not alter the fundamental nature of the reaction.

Measurement of Pulmonary Arterial Pressure.—In accordance with the technique described in our previous paper (2) a small L-shaped glass cannula was passed into the arch of the pulmonary artery without interrupting or obstructing the pulmonary blood flow. This cannula was connected with a water manometer containing red ink and the animal given an intravenous injection of heparin to prevent blood coagulation. In earlier expriments the level of the manometer was read at close intervals but in order to make recording entirely automatic, a Bell Howell 16 mm. motion picture camera was equipped with a small solenoid, operating a plunger, arranged to strike the starting button of the camera. By means of an ordinary laboratory timer the current in the solenoid could be made for an instant at selected and regular intervals, thus providing a series of photographs of the height of the manometer. By including an ordinary watch in the photographic field, strips of film were obtained carrying all the data necessary for plotting the pulmonary pressure curve of the experiment. With the apparatus described photographs could be made as rapidly as sixteen per second. In actual experiment it was found that a photograph every 5 seconds recorded the experiment in ample detail.

Artificial Respiration and the Record of the Onset of Bronchiole Obstruction.—The anterior mediastinal partitions in the guinea pig are widely separated and with a fine dental saw to split the sternum it would undoubtedly be possible to cannulate the pulmonary artery without artificial respiration. But since it was our desire to find out whether the pulmonary vessels contracted during anaphylactic shock as a distinctive phenomenon similar to the contraction observed in the rabbit, and not secondary to the convulsive and asphyxial changes so characteristic of shock in the guinea pig, it was necessary to prevent convulsions and delay asphyxia by the use of curare and artificial respiration. The curare employed was from a lot used in this laboratory in many different series of experiments during the past 4 years and known to be without circulatory effect. For artificial respiration the quantitative respiration pump described by Drinker and Agassiz (6) was utilized. This pump delivers a gentle blast of air for lung inflation and expiration follows passively. In order to identify anaphylactic shock in the guinea pig one must have assurance that interference with the entrance of air into the lungs has occurred and the anesthetized, curarized guinea pig gives no early gross indications of such a situation. If, however, one places a T-tube in the inflow line to the

tracheal cannula from the respiration pump and connects from this T to a tube, the lower end of which is immersed in mercury in a small bottle, an arrangement is provided which records with fair sensitivity any obstruction to the entrance of air in the lungs. In actual practice we used a 90 cc. specimen bottle containing mercury and provided with a rubber stopper pierced by two glass tubes, one long, the other just through the cork. The lower end of the long tube was pushed through the cork and beneath the surface of the mercury just far enough so that no air escaped during the positive stroke by the pump. The shorter tube was connected with a small Krogh spirometer which wrote upon a kymograph. As long as conditions remained constant in the chest of the animal no air entered the spirometer and the writing point made a straight line, but with even very slight obstruction in the respiratory passages, air at once began to overflow into the spirometer and the rise in the writing point signaled the onset of the reaction. In our experiments anaphylactic shock invariably produced a degree of bronchiolar obstruction sufficient eventually to shunt all the air which had been reaching the animal into the overflow spirometer. The rate at which complete deprivation of air took place varied from animal to animal and was never rapid enough to make asphyxia a complicating factor in the distinctive circulatory change seen early in shock.

Sensitization.—All the animals received 2 cc. of sheep serum intraperitoneally and were used for acute experiment from 2 to 5 weeks following this injection.

EXPERIMENTAL.

In the paper (2) describing the technique of pulmonary arterial pressure measurement in the guinea pig, the fact that horse serum given intravenously produces a notable degree of constriction, is described. This is the reaction noted first by Brodie (7) and later explored by Schultz (8) and Edmunds (9). It occurs most markedly in cats. Drinker and Bronfenbrenner (1) found that from a variety of proteins sheep serum proved most innocuous but in some cats even this material gave an occasional reaction. Horse serum, given intravenously to the guinea pig, gives Brodie reactions with somewhat less regularity than in the cat. Sheep serum in doses below 0.3 cc. proved entirely inoffensive and as a consequence animals were sensitized with this material and given a "shocking" injection of 0.1 to 0.2 cc. of the same substance in 1 cc. of physiological saline.

In order to show the great difference between the Brodie reaction and that of anaphylactic shock, a typical experiment of the former type is given.

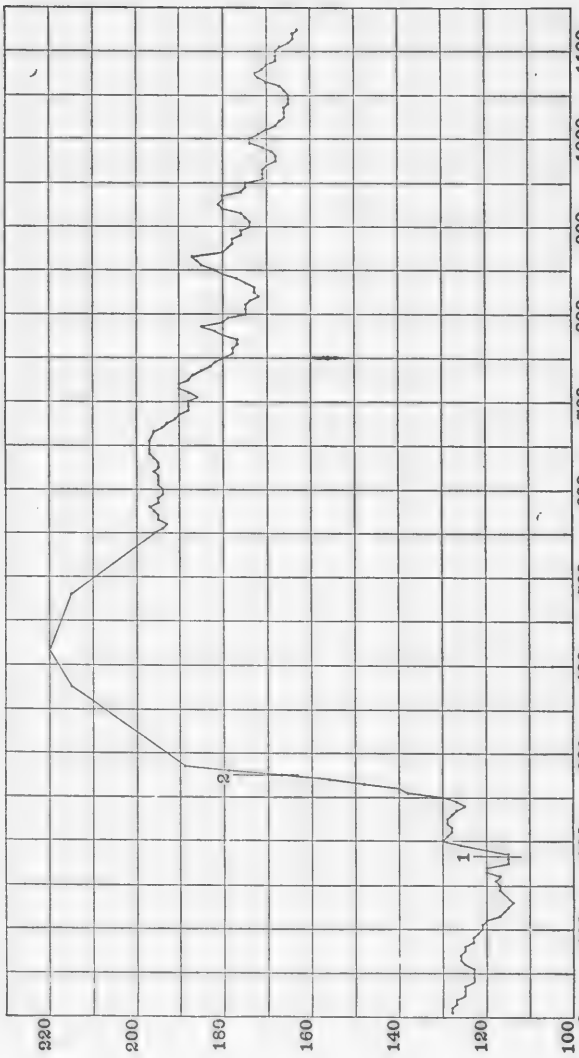

FIG. 1. Pulmonary arterial pressure in a normal guinea pig, which received 1 cc. horse serum intravenously at mark 1. Ordinates, pressure in millimeters of water. Abscissæ, time in seconds. At mark 2, a moderate amount of exclusion of air began.

August 8, 1928. Experiment 1.—Intravenous injection of 1 cc. horse serum. Normal guinea pig. Weight 390 gm. 10:20 a.m. 4 cc. 5 per cent sodium-barbital intraperitoneally. 11:15. Cannulation of external jugular vein and pulmonary artery finished. Animal curarized, heparinized and under artificial respiration with connection to Krogh spirometer. Pressure record begun as shown in Fig. 1. Immediately following the injection of horse serum there is a slight rise in pressure, a volume effect and then an enormous increase which is well sustained. At the mark 2 overflow of air began, indicating difficulty of air entrance into the lungs. This did not increase to a serious degree and is possibly a vascular rather than a bronchiolar phenomenon. The exact vascular site of the Brodie reaction is undetermined. If it is to any degree upon the venous side of the capillary bed the degree of pulmonary congestion produced would readily exclude air as shown by Drinker, Peabody and Blumgart (10) in experiments bearing upon this point.

The contrast between this experiment and those which follow indicates the entire lack of identity between the simple protein and anaphylactic reactions in the guinea pig.

August 9, 1928. Experiment 2.—Intravenous injection of sheep serum in a sensitized animal. Guinea pig, weight 415 gm. Received 2.0 cc. sheep serum intraperitoneally, July 17, 1928. 3:15 p.m. 4 cc. 5 per cent sodium-bartibal intraperitoneally. 4:25 p.m. Cannulation of external jugular vein and pulmonary artery finished. Animal curarized, heparinized and under artificial respiration with connection to Krogh spirometer. Pressure record begun as shown in Fig. 2. At the mark 1, 0.1 cc. sheep serum was given intravenously. At 2 air began to escape through the overflow tube indicating the onset of bronchial obstruction. The fall in pulmonary blood pressure was interrupted momentarily, and then progressed into a long decline accompanied by increasing exclusion of air from the lungs so that by the 400th second the lungs showed practically no movement. At mark 3, the possibility of air overflow was prevented and the artificial respiration adjusted so that a moderate amount of air was entering the lungs, and at mark 4, 1.0 cc. of horse serum was given intravenously. There is the large increase in pressure characteristically due to horse serum, but owing to the poor condition of the animal, not well sustained.

There is a marked contrast between this experiment and the one preceding. A fall in pulmonary arterial pressure characterizes anaphylactic shock in the guinea pig.

August 10, 1928. Experiment 3.—Intravenous injection of sheep serum in sensitized animal. Guinea pig, weight 440 gm. Received 2.0 cc. sheep serum intraperitoneally July 17, 1928. 1:10 p.m. 4.0 cc. 5 per cent sodium-barbital

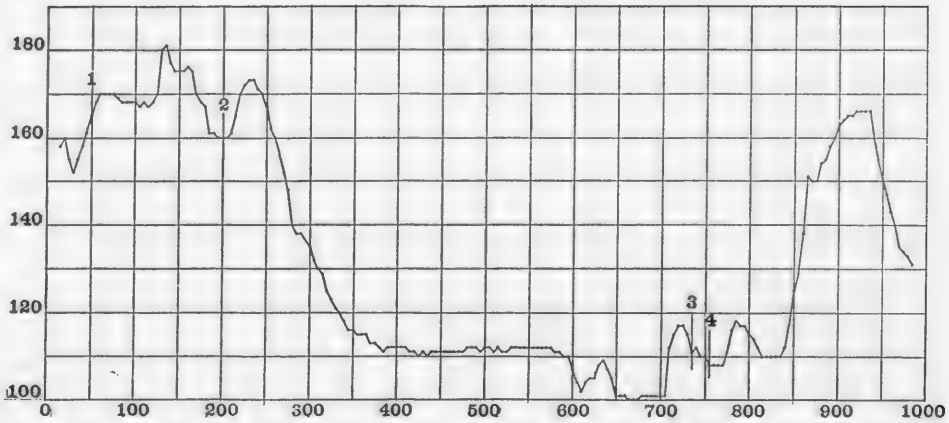

FIG. 2. Pulmonary arterial pressure in a sensitized guinea pig, which received 0.1 cc. sheep serum intravenously at the mark 1. Ordinates, pressure in millimeters of water. Abscissæ, time in seconds. At mark 2 exclusion of air began. At mark 3 air exclusion prevented by forcing overflow tube deeper in mercury. At mark 4, 1.0 cc. horse serum intravenously.

intraperitoneally. 2:04 p.m. Cannulation of external jugular vein and pulmonary artery finished. Animal curarized, heparinized and under artificial respiration with connection to Krogh spirometer. Pressure record begun as shown in Fig. 3. At mark 1, 0.2 cc. of sheep serum was given intravenously. The animal was in very good condition with the pulmonary pressure rising when

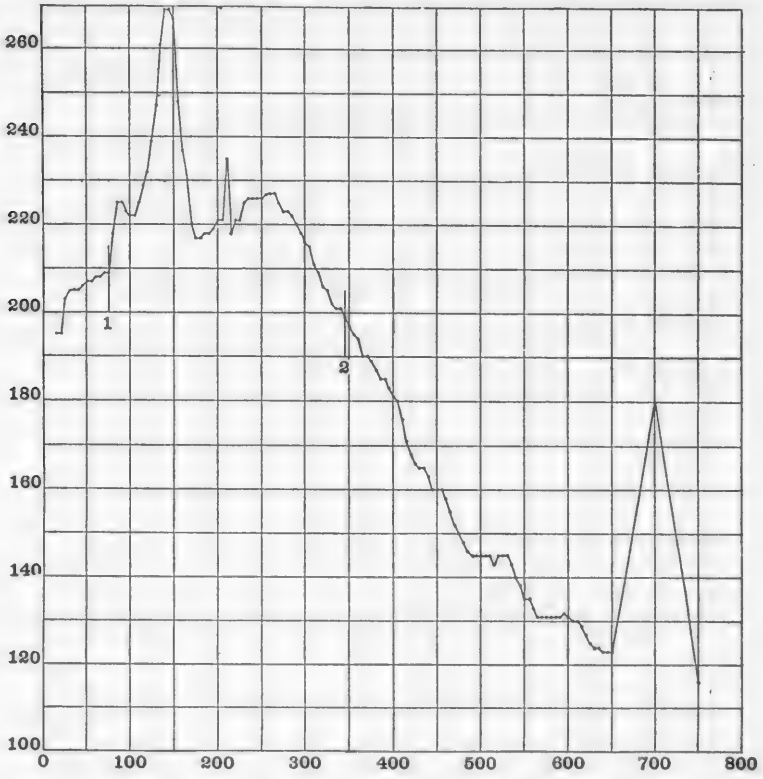

FIG. 3. Pulmonary arterial pressure in a sensitized guinea pig, which received 0.2 cc. sheep serum intravenously at mark 1. Ordinates, pressure in millimeters of water. Abscissæ, time in seconds. At mark 2 exclusion of air began.

the injection was given. At mark 2, air escape into the Krogh spirometer indicated bronchial obstruction, which was rapid and complete. The final rise in pressure is asphyxial and in experiments where systemic pressure was also recorded invariably accompanied the rise in systemic blood pressure, noted by other observers in "shocked" guinea pigs.

August 8, 1928. Experiment 4.—Intravenous injection of sheep serum in a sensitized animal. Guinea pig, weight 310 gm. Received 2.0 cc. sheep serum intraperitoneally July 17, 1928. 3:25 p.m. 6.0 cc. 5 per cent sodium-barbital intraperitoneally. 4:28 p.m. Cannulation of external jugular vein and pulmonary artery finished. Animal curarized, heparinized and under artificial respiration with connection to Krogh spirometer. Pressure record begun as shown

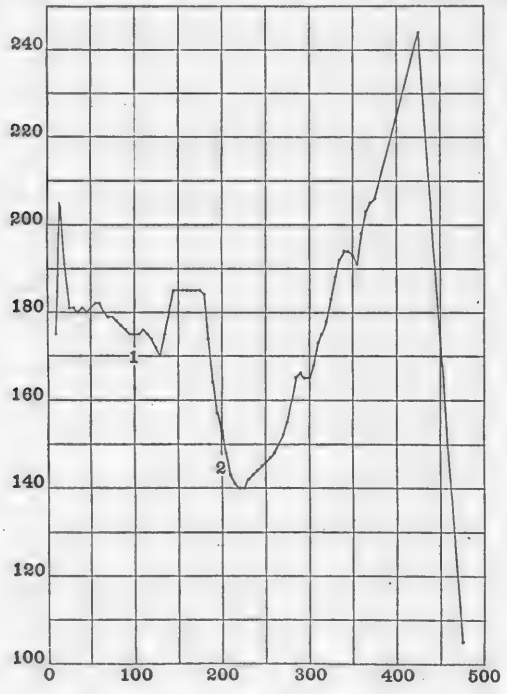

FIG. 4. Pulmonary arterial pressure in a sensitized guinea pig, which received 0.2 cc. sheep serum intravenously at mark 1. Ordinates, pressure in millimeters of water. Abscissæ, time in seconds. At mark 2 exclusion of air began. The large rise in pressure is the effect of terminal asphyxia.

in Fig. 4. At mark 1, 0.2 cc. of sheep serum was given intravenously and at mark 2 bronchial obstruction was recorded. This condition became complete almost immediately as noted by the subsidence of lung movement. Asphyxial rise in pulmonary pressure began promptly and could be but slightly checked by adjusting the respiration pump so as to attempt to force air into the lungs. The final drop in the curve occurred just before death.

August 8, 1928. Experiment 5.—Intravenous injection of sheep serum in a sensitized animal. Guinea pig, weight 450 gm. Received 2.0 cc. sheep serum intraperitoneally July 17, 1928. 3:18 p.m. Anesthesia and preparation identical with former experiments. Shocking injection of 0.2 cc. sheep serum given intravenously at mark 1, Fig. 5, and at mark 2 bronchial obstruction was recorded. There was the usual fall in pulmonary blood pressure followed by a slight asphyxial rise before the death of the animal.

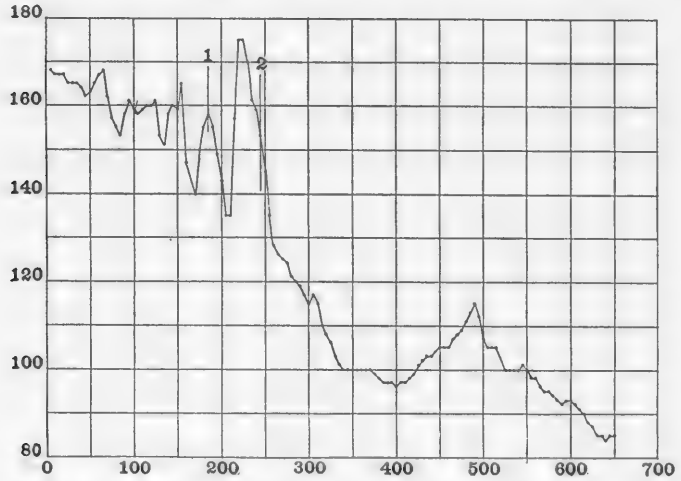

FIG. 5. Pulmonary arterial pressure in a sensitized guinea pig, which received 0.2 cc. sheep serum intravenously at mark 1. Ordinates, pressure in millimeters of water. Abscissæ, time in seconds. At mark 2 exclusion of air began. The secondary rise in pressure is asphyxial and is followed by a decline leading to the death of the animal.

DISCUSSION.

The experiments cited show that as a very early phase of the anaphylactic disturbance there is a fall in pressure in the pulmonary circuit. Given a normal heart, and all observations of anaphylaxis in the guinea pig as well as in other animals[1] indicate minimal effects upon the actual pumping power of the heart; the great factor affecting

[1] Drinker and Bronfenbrenner (1) showed that the serious effects of anaphylactic shock on the heart of the rabbit were secondary to pulmonary obstruction and not a primary ventricular phenomenon of distinct importance as postulated by Auer (11).

pressure in the pulmonary circulation is the flow of blood into the right ventricle. The fall in pulmonary pressure observed in our experiments would thus appeal to the physiologist as due to some reaction in the animal which interfered with cardiac filling. This is exactly what happens in the dog, where owing to interference with blood flow through the liver, blood pools in the abdominal vessels, the pulmonary pressure falls and as the left ventricle begins to receive less blood the systemic pressure drops sharply.

But neither in our experiments nor in those of others has there been any fall in systemic blood pressure, and while the criterion is not absolutely delicate, it is sufficiently so to cause us to feel that the fall in pulmonary pressure is more probably due to a gradual widening of the pulmonary vascular bed with an increase in the pulmonary blood volume not great enough to fail of compensation by vasoconstriction on the systemic side of the circulation, and so unaccompanied by fall in systemic blood pressure.

Examination of Figs. 2 to 5 shows that with the exception of Experiment 2 and in this it would seem as if the characteristic fall in pulmonary pressure is but broken temporarily by a slight rise, the pulmonary vascular reaction precedes evidence of bronchiolar obstruction. Possibly the bronchiolar effect, so prominent in the guinea pig, may thus have a secondary relation to this vascular dilatation.

SUMMARY.

1. Sheep serum in doses below 0.3 cc. intravenously produces no pulmonary vasoconstriction in the guinea pig. Guinea pigs have consequently been sensitized with this substance and anaphylactic shock produced by doses of 0.1 and 0.2 cc.

2. Pressure in the pulmonary artery has been measured by the method of Drinker and Went (2) and recorded photographically in a new and convenient manner.

3. At a very early stage in anaphylactic shock the pulmonary arterial pressure falls markedly and this fall seems to precede the appearance of bronchiolar obstruction.

4. The fall in pulmonary blood pressure in anaphylactic shock is in marked contrast to the rise in pressure resulting from intravenous injection of toxic foreign protein, such as horse serum.

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STUDIES ON HYPERSENSITIVENESS TO DIPHTHERIA BACILLI.

I. An "Immediate" Skin Reaction Which Can Be Passively Transferred.

By JAMES M. NEILL, Ph.D., and WILLIAM L. FLEMING.

(*From the Department of Bacteriology and Immunology, Vanderbilt University Medical School, Nashville.*)

Plates 2 to 5.

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Hypersensitiveness of man to bacteria is of general importance because of its unknown effects upon infection and resistance and upon the manifestation of disease. From at least one aspect, hypersensitiveness to diphtheria bacilli seems to be especially important. While there are few diseases in which the manifestations can be referred so directly to the toxin, there are few pathogenic bacteria that are as widely distributed as the diphtheria bacillus. The question of hypersensitiveness to these bacteria, therefore, must be considered significant for it can be assumed that most city adults have probably had at some time in life such opportunities to become sensitive as may be afforded by contact with diphtheria bacilli.

The present paper describes an "immediate" skin reaction to diphtheria bacilli, and presents experimental evidence to establish it as a true hypersensitive phenomenon distinct from the "delayed" skin reaction commonly encountered in Schick tests on adults ("pseudo-reaction").

The common division of human skin reactions into "immediate" and "delayed" reactions (as reviewed in detail by Zinsser (1) and others), seems to present a separation of two fundamentally different types of antibacterial hypersensitiveness. In general, the hypersensitive condition manifested in man by "immediate" skin reactions has much in common with the condition of "true anaphylaxis" (Wells (2)) obtained in the guinea pig by laboratory sensitization. In view of the infrequent reports of the "immediate skin reaction type" of hypersensitiveness in

man to bacteria, our interest was aroused when we obtained an "immediate" skin reaction in an apparently normal adult to the bacterial derivatives contained in the routine Schick test material. Since Schick tests are employed so frequently, it seemed to us that if this type of hypersensitiveness were not rare it must have been observed in the past. A thorough review of the literature, however, revealed no reports of hypersensitive skin reactions that could be referred definitely to the diphtheria bacillus itself, other than the well known "pseudoreaction" which is a classical example of the "delayed skin reaction type" of hypersensitiveness.

The "Immediate" Skin Reaction.

The "immediate" skin reaction reported in this paper was first observed in March, 1926, on one individual in a group of 40 medical students who had received the routine Schick test. The "immediate" and the "delayed" reactions exhibited by this man are described below:

"Immediate" Reaction.—Within 5 minutes after the injection of the Schick test material, the man experienced a slight itching on the areas of the injections. Within 15 minutes, a blister about 25 mm. in diameter, with definite pseudopodia became evident; the blister was surrounded by a solid zone of bright red erythema extending out 20 to 25 mm. with punctiform, scattered points of erythema extending beyond the solid zone. This reaction is essentially the same in appearance as that often observed in the common examples of the "immediate skin reaction type" of hypersensitiveness, and requires no further description since pictures of the reaction are given in Figs. 1 and 2. The reactions were essentially the same on the left arm which had been injected with heated (75°C.) Schick toxin as on the right arm which had been injected with unheated Schick toxin.

This immediate reaction began to fade within 1 hour after the intradermal injection and disappeared within 2 hours.

"Delayed" Reactions.—After the disappearance of the "immediate" reaction (Fig. 3), the further manifestations of this individual's reaction to diphtheria filtrate were the same as that frequently observed in adults who give pronounced "combined reactions" in the routine Schick test. The "combined reaction" (Fig. 4) is too well known to require a detailed description of the "delayed" reactions in this particular individual.

The individual giving the "immediate" skin reaction to diphtheria filtrate was 28 years of age. His diphtheria history is indefinite. It is possible that he had a mild attack when about 4 years old, since he experienced a sore throat at the time of a diphtheria epidemic in Corfu. He was not severely sick, but was kept in bed for 3 days by the physician. No cultures were taken. No antitoxin was given. The sore throat was not diagnosed as diphtheria by the physician in spite of the local prevalence of the disease.

He has never been immunized with toxin-antitoxin or toxoid. There is no evidence that he is a carrier; negative results were obtained in all attempts to detect diphtheria bacilli either by staining or by guinea pig inoculation of Loeffler's serum cultures made at different times from his throat, nose, and ears. Skin tests with a wide variety of substances seemed undesirable during our investigation but, as far as clinical symptoms are concerned, he has no recognizable asthma, hay fever, or food idiosyncrasies. There is, thus, no evidence that this individual has been endowed with an unusual tendency to become sensitized or that he has had any greater opportunities to become sensitive to diphtheria bacilli than have many other city adults.

Tests for "immediate" skin reactions to diphtheria filtrate (0.0008 cc.) were negative in all the immediate relatives of the diphtheria-hypersensitive individual; namely, the mother (60 years old), a sister (26 years old), and her three children (2 to 5 years old). No clinical symptoms of hypersensitiveness (asthma, hay fever, food idiosyncrasies) have ever been observed in any of them.

Comparison of the Skin Reactions Induced by Broth.

The material injected in the Schick test is the diluted filtrate of a broth culture of the diphtheria bacillus. Experiments were made to determine whether or not the broth culture medium itself was responsible for the "immediate" skin reaction. The first tests were made with the same amount of broth as that contained in the dose of the culture filtrate utilized in the Schick test with which the reaction was first observed. A sample of the same broth culture medium as that employed at the Massachusetts Antitoxin Laboratory in the preparation of this particular lot of Schick toxin was obtained. The equivalent amount (0.0004 cc.) of the broth contained in 0.1 cc. of salt solution was injected and found to elicit no "immediate" skin response at all.

It seemed important, however, to compare the response of the apparently sensitive individual with the response of other individuals to larger amounts of broth than that contained in the Schick test dose. The tests were made on 12 other normal individuals, with doses of 0.02 cc and 0.001 cc. of broth. The larger amount (equivalent to 50 times the amount injected in our Schick test dose) gave a pronounced "immediate" skin reaction in the test individual and in 11 out of 12 of the control group. The reaction was of the same appearance as that usually obtained in the "immediate skin reaction type" of hypersensitiveness; it is probably not a hypersensitive reaction at all, but only the normal response to a constituent of broth which is primarily toxic in man when injected in such large amounts. The smaller dose (equivalent to 2.5 times the Schick test dose) gave no reaction in the test individual although it did give small but definite reactions in a few of the group; consisting of an erythematous rash, without definite raising of the wheal and without pseudopodia. With neither dose of broth, however, was the reaction of the test individual greater than that of the majority of the normal individuals in the control group.

The failure of the hypersensitive individual to give any "immediate" skin response to the amount (0.0004 cc.) of broth equivalent to that contained in the Schick test dose, together with his failure to give quantitatively greater reactions to larger amounts of broth than those given by a group of other individuals, is important; for it shows that he is neither truly "hypersensitive" to broth nor does he possess a low degree of "tolerance" to broth constituents which apparently are primarily toxic to other men when injected intradermally in sufficient quantity.

"Immediate" Skin Reaction to Washed Diphtheria Bacilli (Heated) and to Sterile Filtered Extracts.

Experiments were made to determine whether the "immediate" skin reaction could be produced by diphtheria bacilli when washed free from all traces of culture fluid. Two sorts of material were tested: (1) salt solution suspensions of the washed bacterial cells; (2) sterile solutions or extracts of the intracellular substances derived by repeated freezing and thawing of concentrated suspensions of the washed bacilli.

Preparation of Bacterial Suspensions.—Broth cultures (5 days old) of the Park 8 strain of the diphtheria bacillus were centrifuged and the bacilli resuspended in salt solution. They were then washed 3 times with 50 cc. of sterile salt solution; the washing removed all traces of broth remaining on the bacilli, for the centrifuged sediment was sufficiently compact to enable one to remove, after each washing, practically all traces of the supernatant fluid. The washed bacteria were finally suspended in a volume of salt solution equivalent to the original broth culture, heated for 15 minutes at 80°C., and 0.4 per cent of phenol added. Sterility was tested by culture in glucose broth, and by intradermal injection into guinea pigs.

Preparation of Solutions (Extracts) of the Intracellular Substances.—The bacterial cells from a broth culture of a recently isolated strain of the diphtheria bacillus were collected and washed as described above, and finally resuspended in a volume of salt solution equivalent to 1/20 the original volume of broth culture. The concentrated suspension of washed bacterial cells was then repeatedly frozen and thawed as described in a preceding paper for the preparation of diphtheria bacillus enzymes (3). The frozen and thawed suspensions were finally filtered through a Berkefeld candle. These extracts or solutions contained a certain amount of intracellular substances as demonstrated by a slight but definite coagulum in boiled samples of the solution and by the presence of the active maltase enzyme which is known to be an endocellular constituent of the diphtheria bacillus (3). The extracts were not heated, but were proved sterile by culture and by guinea pig inoculation.

The tests for "immediate skin reaction" were made by the intradermal injection of 0.1 cc. volumes of salt solution containing: (1) heated suspensions of washed bacterial cells equivalent to the bacteria contained in 0.0004 cc. of the broth culture; (2) 0.00005 cc. of the sterile filtered extract of the frozen and thawed suspension of washed bacilli. Salt solution was injected as control.

The results of the tests with the washed bacilli and with the sterile filtered extract prepared by physical disintegration of the washed bacilli, showed that these substances induced the same type of "immediate" skin reaction as had the filtrate of the broth culture. Similar injections in 25 other individuals gave no evidence of any "immediate" skin response to washed diphtheria bacilli. This experiment, together with the preceding one, indicated that the described "immediate" reaction was due to some constituent of the diphtheria bacillus itself, contained in broth culture filtrates, in suspensions of the washed bacterial cells, and in filtered solutions of substances derived from the washed bacteria.

The results with the washed bacilli are also of interest as proof that neither active toxin nor its heat-inactivated derivatives are involved in the hypersensitive reaction. The suspension of washed bacilli can be assumed to have been freed from soluble toxin by washing the cells, and since they were heated at once, the bacilli were killed before new toxin could have been formed if it were possible to do so in salt solution. The bacterial extracts, while unheated, were likewise devoid of amounts of toxin detectable in guinea pigs, 2.0 cc. of the undiluted and unheated extract injected subcutaneously being innocuous, and the intradermal injection of 0.15 cc. failing to give a skin reaction on normal guinea pigs. These tests for the presence of toxin show that the amount of the bacterial extract (0.00005 cc.) which gave a strong "immediate" reaction in the hypersensitive individual must have contained an absurdly small amount of toxin, if any. (The amount (0.15 cc.) injected intradermally in the guinea pig without reaction was 3,000 times the amount injected in the hypersensitive man. Since 1/500 M.L.D. is commonly accepted as giving a definite skin reaction in a guinea pig (4), then the amount of toxin contained in the unheated extract that produced the "immediate" hypersensitive reaction must have been less than 1/1,500,000 M.L.D.)

The general question of toxin hypersensitiveness is of considerable theoretical interest and although considerable work has been done in the past, the question is still an open one (5). In the present instance, however, there is no reason to believe that the toxin or any of its heat-inactivated derivatives have anything at all to do with the hypersensitiveness of the individual described in this paper.

The Bacterial Specificity of the "Immediate" Skin Reaction.

The first experiments were made with heated suspensions of washed bacterial cells and consisted of a comparison of both the "immediate" and the "delayed" reactions induced by the intradermal injection of diphtheria bacilli with the reactions invoked by other bacteria. Colon bacilli, typhoid bacilli, and Type I pneumococci were used as species not closely related to diphtheria bacilli; and *hofmanni* and xerosis bacilli as bacteria supposed to be more closely related to the diphtheria organisms. In order to make the test of the specificity more rigid, the amount of bacterial substance was made twice as great in the test doses of the other kinds of bacteria as in the test dose with the diphtheria bacilli. The doses were based upon the bacterial substance contained in the suspensions as determined by turbidity comparisons.

Preparation of Bacterial Suspensions.—Broth cultures (18 hours old in the case of the pneumococci and 5 days old in the case of the other bacteria) were centrifuged, the bacterial cells washed 3 times with large volumes of sterile salt solution, and finally resuspended in salt solution in a volume equivalent to that of the original broth culture. The bacteria were killed by 15 minutes exposure to 80°C. and the sterility of the suspensions checked by cultural tests. Since the growth of the different bacteria was not comparable, all of the suspensions were adjusted to a turbidity equivalent to that of a 1/20 dilution of the original diphtheria bacillus suspension. The adjusted suspensions, after the addition of 0.4 per cent phenol, were used as stock suspensions from which the test doses were prepared by their proper dilution.

Test Injections.—The tests themselves were made by intradermal injection of 0.1 cc. of suspensions of the different kinds of bacteria in different areas of the flexor surface of the forearms. The dose of diphtheria bacilli was equivalent to the bacteria contained in 0.0005 cc. of its broth culture; the amount of the other kinds was equivalent to twice the bacterial substance contained in the diphtheria test dose. Observations at 5 minute intervals for 1 hour were made for the "immediate" reactions; and observations at 4 hour intervals for 24 hours for the "delayed" reactions. Two normal individuals were injected at the same time with the same materials.

The results of two experiments made at different times were approximately the same. The "immediate" reactions are shown in Fig. 5.

The results of these experiments (Fig. 5) showed that the "immediate" reaction to diphtheria bacilli is specific and not invoked by all bacteria regardless of species. Although the bacterial substance was twice as great as in the diphtheria test, pneumococci, *hofmanni* bacilli, typhoid bacilli, and colon bacilli gave no "immediate" skin reaction at all. The xerosis[1] bacilli, on the other hand, gave a definite "imme-

diate" reaction, weaker yet of the same type as that invoked by the smaller amount of diphtheria bacilli. The reaction given by the xerosis bacilli does not detract at all from the specificity of the diphtheria reaction and in fact, is important in itself as an index of a probable immunological group relationship between our strain of xerosis and the usual diphtheria bacillus.

The fact that some of the different bacteria gave "delayed" reactions in this man and others did not, is not of particular importance in this paper, for the majority of adults give "delayed" reactions to some kinds and not to other kinds of bacteria. But it is important that the xerosis bacilli which gave a definite "immediate" reaction failed entirely to cause a "delayed" reaction. This furnishes a sharper distinction between the "immediate" and "delayed" skin reactions than can be obtained with diphtheria bacteria in this individual because a dose of diphtheria which gives only a small "immediate" reaction still gives a definite "delayed" reaction.

The following experiments differed from the preceding ones chiefly in the fact that filtrates of broth cultures of the different kinds of bacteria were employed instead of suspensions of the washed bacterial cells.

In the preceding experiments, the dose of the other bacteria was twice the diphtheria dose, while in the present ones, the amount of the filtrate of the other kinds of bacteria was increased to 5 times that of the diphtheria filtrate. The increase in the relative dose of the filtrates of the other bacteria made the test for specificity more rigid, but was chosen particularly in order to determine if the "immediate" reaction to the xerosis filtrate could be made comparable in size to the "immediate" reaction to the diphtheria filtrate without producing a "delayed" skin reaction.

Two experiments were made with similar results, which are presented in Table I. Pictures of the "immediate" and "delayed" reactions are shown in Figs. 6 and 7.

The results (Table I and Figs. 6 and 7) of the experiments with the filtrates were essentially the same as those obtained with the suspensions of washed bacterial cells. But the results with the filtrates are of additional interest as evidence that the specificity of the "immediate" reaction holds true even when the amount of culture filtrate of the unrelated bacteria (typhoid, colon, *hofmanni* bacilli, and pneumococci) is 5 times as great as the diphtheria dose.

TABLE I.

Bacterial Specificity of the "Immediate" Skin Reaction in Tests with Broth Culture Filtrates of Different Kinds of Bacteria.

Broth culture filtrates		"Immediate" reactions (20 min. after time of injection)		"Delayed" reactions (24 hrs. after time of injection)	
Kind of bacteria	Amount of filtrate				
	cc.				
Diphtheria bacilli	0.0001	Strong	30 × 35 mm. intensely red, solid area; surrounded by additional slight speckling	Strong	20 × 30 mm. intensely red and slightly swollen area; increased since 16 hr. reading
Xerosis bacilli	0.0005	Strong	35 × 40 mm. intensely red, solid area; slightly greater than the reaction to the smaller amount of diphtheria	None	No redness and only faint mark of needle point
Typhoid bacilli	0.0005	None.	Indistinguishable from broth control	Strong	15 × 20 mm. moderately red and slightly swollen area; reduced since 16 hr. reading
Colon bacilli	0.0005	None.	Indistinguishable from broth control	Strong	15 × 20 mm. moderately red and slightly swollen area; reduced since 16 hr. reading
Pneumococci	0.0005	None.	Indistinguishable from broth control	None	No redness and only faint mark of needle point
Hofmanni bacilli	0.0005	None.	Indistinguishable from broth control	None	No redness and only faint mark of needle point
Broth control	0.0005	None	Only a faintly pink area (5 × 6 mm.) at site of original injection; a faint trauma reaction	None	No redness and only faint mark of needle point

The most significant evidence produced by these experiments, however, consists of the relations between the skin reactions to diphtheria and the reactions to xerosis. In the previous experiment, it was shown that with the doses used the xerosis and diphtheria washed bacilli were alike in that both produced "immediate" skin reactions but differed in that only diphtheria produced a "delayed" skin reaction. The present experiment (Table I) showed that by changing the ratio of the xerosis and diphtheria doses the "immediate" reaction to the xerosis filtrate was made larger than the "immediate" reaction to the diphtheria filtrate; and that even under these conditions, the xerosis filtrate failed to cause the "delayed" skin injury produced by the smaller dose of diphtheria filtrate. These relations indicated that while both "immediate" and "delayed" reactions were produced by diphtheria bacteria or filtrate, they were due to different constituents of this bacillus. The substance responsible for the "immediate" reaction was also possessed by our strain of xerosis bacilli. The constituent responsible for the "delayed" reaction was either not possessed by the xerosis bacilli or the two substances were present in them in a ratio much different from the ratio in which they were present in diphtheria bacilli since a dose of xerosis material which failed to produce a "delayed" reaction produced a larger "immediate" reaction than did a dose of diphtheria material which also produced a pronounced "delayed" reaction.

The production of "immediate" reactions by the xerosis bacilli and filtrates presents further evidence that diphtheria toxin is not involved in the hypersensitiveness. This strain of xerosis although immunologically related to diphtheria bacilli is absolutely non-toxicogenic and is avirulent for guinea pigs and rabbits. A number of both kinds of animals have been injected with 10 cc. of broth culture, and rabbits have been injected with suspensions containing the concentrated bacilli from 750 cc. of broth culture with apparently no harmful effect.

The Influence of Heating the Filtrate.

The filtrate of a 7 day broth culture was diluted 8 times with sterile salt solution and then divided into 3 equal portions. The first portion was heated for 10 minutes at 80°C. as in the usual inactivation of the heated control used in the Schick test; the second portion was heated at 100°C. for 1 hour; and the third at 120°C. for 30 minutes. Phenol in physiological salt solution was then added so that the filtrate was finally diluted 10 times in salt solution containing 0.4 per cent

phenol. (The phenol was added last in order that it might not be present during the heating processes.)

In the particular experiment presented in Figs. 8 and 9 the test doses employed were 0.0004 cc. of filtrate, which is as much as that usually contained in the Schick test dose. The same amount of the same broth as that utilized in growth of the culture was injected as the control.

The results (Figs. 8 and 9) of these experiments were always the same: exposure of diphtheria culture filtrate to temperatures above 80°C. had a much greater effect upon its ability to induce the "immediate" reaction than upon its ability to invoke the "delayed" reaction. Heating the filtrate at 100°C. for 1 hour weakened its reactivity in respect to the "immediate" reaction but had no detectable effect upon the "delayed" reaction; exposure to 120°C. for 30 minutes seemed to destroy its ability to induce the "immediate" reaction, but caused only a quantitative decrease in the "delayed" reaction. These results present further evidence that the "immediate" and the "delayed" reactions represent two distinctly different types of hypersensitive phenomena, the "immediate" response being brought about by a diphtheria constituent which is more heat-labile than is the constituent responsible for the delayed injury of the usual "pseudoreaction" of the Schick test.

The relative heat labilities of the substances involved in the two types of reactions were confirmed with other test doses as well as with the dose (0.0004 cc. of filtrate) used in the described experiment.

The amount of the filtrate which failed to give any "immediate" reaction in the described experiment after it had been heated at 120°C. is at least 10 times as great as the minimum amount required to give a definite reaction to the same filtrate which had been heated at only 80°C. It was impossible to determine whether the inactivation at 120°C. was complete to the extent that much larger amounts would still fail to give any "immediate" reaction, because of the marked and somewhat painful "delayed" reactions given in this individual by large doses of the filtrate even after this treatment.

The Passive Transfer of the "Immediate" Skin Reaction.

Attempts to passively sensitize local skin areas (6) in the arms of normal individuals were made by the intradermal injection of the serum of the person who gave the "immediate" skin reaction to diphtheria. The serum of another individual who gave a pronounced "delayed" but no "immediate" reaction to diphtheria,

was also injected into another local area in order to determine whether or not the "delayed" reaction could be passively transferred. The tests of the passive transfer were made 48 hours after the introduction of the serum, and consisted of the injection of equal doses of diphtheria filtrate into each of the areas prepared by previous injection of the two foreign sera and into a control area previously injected with sterile salt solution. Although most of the individuals were tested with diphtheria broth culture filtrate, some were tested with heated salt solution suspensions of the washed bacilli.

During our investigation we have made transfer tests with over 100 normal individuals and a detailed analysis of the results will be given in a following paper. It is desired in the present paper to present simply the fact that the "immediate" skin reaction was passively transferred to a significant percentage of the individuals of the group; and that the "delayed" reaction failed to be transferred to a single one of the large group tested. The "immediate" reactions in the passively sensitized skin areas of the normal people when injected either with filtrate (Fig. 10) or washed bacilli (Fig. 11) were of significant dimensions and intensity and qualitatively of exactly the same appearance as those shown by the donor of the serum when injected himself with diphtheria material.

The Bacterial Specificity of the Passive Transfer.

Experiments to determine whether or not the bacterial specificity of the "immediate" reaction held true in tests on passively sensitized people were made by sensitizing a number of areas on the arms of normal individuals and subsequently injecting them with equal amounts of the filtrates of different kinds of bacteria.

The results (Fig. 12) of these experiments which were the same with the 4 normal individuals tested, showed that the reaction was just as specific in passively sensitized individuals as in the hypersensitive individual; *i.e.*, the filtrates of the bacteria apparently unrelated to diphtheria bacilli (typhoid, colon, *hofmanni* bacilli, and pneumococci) gave no "immediate" reactions, while the xerosis filtrate gave a definite "immediate" reaction which was of the same type, but less in intensity and extent than that invoked by diphtheria filtrate. The "immediate" reaction to xerosis was less in comparison to the "immediate" reaction to diphtheria filtrate in the passive transfer experiments (Fig. 12)

than in the previous tests with the same filtrates on the hypersensitive individual himself (Fig. 6); but this is due simply to the fact that equal doses of diphtheria and xerosis were used in the passive transfer experiments, while in the previous experiments with filtrates on the hypersensitive man the xerosis dose was 5 times greater than the diphtheria dose.

The Influence of Heating Treatment of Diphtheria Culture Filtrate upon "Immediate" Reactions in Passively Sensitized Skin Areas.

The influence of previous heating treatment of diphtheria culture filtrate upon its capacity to produce "immediate" reaction in passively sensitized skin areas was studied by injecting equal doses of filtrate heated at different temperatures into different locally sensitized areas of a normal individual.

The results (Fig. 13) were essentially the same as in the analogous experiment (Fig. 8) upon the diphtheria-sensitive individual—*i.e.*, 1 hour's heating of the diphtheria filtrate at 100°C. caused a marked decrease in, and 30 minutes at 120°C. caused an apparently complete loss of, the property of producing "immediate" skin reactions in locally sensitized areas on normal individuals. The fact that previous heating treatment of diphtheria culture filtrate had the same effect upon its property of producing "immediate" skin responses in passively sensitized individuals as in the diphtheria-hypersensitive man, is important as evidence that the same bacterial constituent is responsible for the skin reaction in both instances.

Other Experiments upon the Passive Transfer.

One passive sensitization sufficed in the more responsive normal individuals for the production of another "immediate" reaction to a second injection of the same dose of diphtheria filtrate made about 1 week after the production of the first "immediate" reaction. The second injection was not always successful, but usually was in individuals who gave pronounced reactions to the first injection.

Most of our tests have been made 48 hours after the injection of the sensitive serum. This time was chosen partly for convenience and partly in order to allow all traces of the serum injection or of trauma, to disappear from the passively prepared skin areas. A longer time can elapse between the injection of the sensitive serum and the injection of the bacterial filtrate, for in the only individual tested, a marked "immediate" reaction was manifested when the filtrate was injected 6 weeks after the injection of the serum.

DISCUSSION.

This paper reports a study of a hypersensitive condition toward diphtheria bacilli, which is not common in man. The hypersensitiveness was first observed by the "immediate" skin reaction invoked in an individual by the intradermal injection of the diluted broth culture filtrate utilized in the routine Schick test. This skin reaction (Figs. 1 and 2) seems to be identical with the "immediate" reactions commonly produced by the intradermal injection of horse serum or of ragweed pollen in individuals sensitive to those substances. It is invoked not only by filtrates of broth cultures but also by heat-killed suspensions of the bacilli freed of broth and toxin by thorough washing, and by solutions of the bacterial substances liberated when washed diphtheria bacilli are partially disintegrated by repeated freezing and thawing. The Park 8 strain of diphtheria bacilli was used in all but one of the experiments reported in this paper. However, in other tests "immediate" reactions of the same type have been produced by a number of other strains of different origin.

This skin manifestation is specific and is not invoked by unrelated bacteria, for the diphtheria-sensitive individual does not give any "immediate" reaction either to the washed bacterial cells or to the broth culture filtrates of typhoid, colon, *hofmanni* bacilli, or pneumococci, even when the amount of bacterial substance is much greater than that required for pronounced reactions with diphtheria bacteria. Reactions of the same quality are invoked by our strain of xerosis[1] bacteria (either by washed bacilli or by broth culture filtrates) but larger amounts are required to give reactions of the same extent and

[1] In this paper we have used "*xerosis bacillus*" as a convenient term to refer to the particular strain used in our experiments and we wish to emphasize the fact that there is no reason to believe that immunological properties possessed by it would be common to all diphtheroids included in the loose use of *C. xerosis* in the species sense. The strain used in our experiments was of unknown origin; it agrees with the strain of *C. xerosis* in the American Type Culture Collection, in respect to the properties (sugar fermentations, etc.) generally used in the separation of the diphtheria group; we have not tested the latter strain to determine whether or not it will cause the "immediate" skin reaction. However, we have isolated a diphtheroid from the individual reported in this paper; and although it agreed in sugar fermentations with the usual definition of *C. xerosis*, it failed to produce the "immediate" skin reaction.

intensity as those produced by diphtheria bacilli (Fig. 6). The "immediate" reaction to xerosis is not an argument against the bacterial specificity of the hypersensitive skin reaction, but in all probability is an index of an immunological group relationship between our strain of xerosis and the usual diphtheria bacillus.[2] The diphtheria bacterial substance which is responsible for the "immediate" skin reaction is relatively heat-labile. While exposure of the filtrate to 80°C. for 10 to 20 minutes does not materially diminish its capacity to invoke the reaction, exposure to 100°C. for 1 hour markedly lessens this capacity and exposure to 120°C. for 30 minutes seems to destroy it entirely (Fig. 8). Previous heating treatment of xerosis culture filtrate had the same effect, which indicates that the bacterial substance responsible for the "immediate" skin reaction to xerosis has the same degree of heat lability as has the kindred substance of diphtheria bacteria.

One of the most important characteristics of the described bacterial hypersensitiveness is its passive transfer to local areas of the skin of normal individuals by the intradermal injection of the diphtheria-sensitive individual's serum. After the hypersensitiveness has been passively transferred, manifestations of the same character and appearance are invoked by the same materials in the locally sensitized skin areas as in the skin of the hypersensitive individual himself. That is, the "immediate" skin reaction is produced either by broth culture filtrate (Fig. 10) or by washed diphtheria bacilli (Fig. 11); to a less extent by xerosis bacilli or their culture filtrates (Fig. 12), but not at all by broth or by the culture filtrates or washed cells of unrelated

[2] Our explanation of the "immediate" skin reaction to both xerosis and diphtheria upon the basis of an immunological relationship between these two kinds of bacteria is not an unjustified one. We have found in experiments to be published later, that there is a serological relationship between our strain of xerosis and at least some strains of diphtheria bacilli, and hence, there is reason to believe that an individual sensitized by one of these bacteria might manifest hypersensitiveness toward both of them. The fact that larger amounts of the xerosis bacilli are required to give the "immediate" reaction may be due either to a lower concentration of an antigen identical with the particular one of the diphtheria bacilli or to a somewhat less degree of reactivity of a xerosis antigen which is related to but not identical with the one contained in the diphtheria bacilli.

Since the conditions of acquisition of the hypersensitiveness are entirely un-

bacteria (Fig. 12); and is similarly affected by previous heating treatment of the diphtheria filtrate (Fig. 13). All these facts furnish convincing evidence that identical bacterial substances are responsible for the "immediate" reaction whether it is invoked in the skin of the diphtheria-hypersensitive man or in the locally sensitized skin areas of normal men.

The described hypersensitiveness to diphtheria bacilli is not a transient condition in this individual. The "immediate" skin reaction has been manifested in tests covering a period of 28 months with no appreciable differences in intensity. His serum has likewise retained its capacity to transfer the hypersensitiveness with no detectable difference in the various samples of serum obtained in bleedings made at different times during 18 months.

Broth is not in any way responsible for the "immediate" reaction of this man to diphtheria culture filtrate. Skin reactions of the same type can be elicited in some people by small amounts of broth and in most normal individuals by large amounts, but this particular man does not respond at all to the small amount contained in the test doses of filtrate nor does he exhibit a less degree of "tolerance" to large doses of broth than do the majority of normal adults. Further facts to eliminate broth are furnished by the reactions induced by the washed bacilli and solutions prepared from washed bacilli; and by the heat (120°C.) stability of the substance responsible for the "immediate" skin reactions in the individuals who do give pronounced reactions to broth.

In view of the theoretical importance of the mooted question (7) of

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known, there is no proof that the "immediate" reaction to xerosis as well as to diphtheria is not due to individual sensitization with both these bacteria. It is simpler, however, to assume that he has been sensitized with only one of them, for it would be an improbable coincidence for this particular man to have become sensitized at different times with two different bacteria that are serologically related in the antiserum of rabbits and to have failed to have become sensitized against the other unrelated, but common bacteria that we have tested. The fact that with dosage controlled, he gives a larger "immediate" reaction to diphtheria than to xerosis bacterial material, together with the fact that he gives a pronounced "delayed" ("pseudo") reaction to diphtheria, makes it more likely that diphtheria rather than xerosis bacilli were the sensitizing agents.

toxin hypersensitiveness, considerable attention was given to the possibility that toxin might be involved in the diphtheria hypersensitiveness of this individual. However, the active toxin is not involved at all, for heated (80°C.) filtrates in which the toxin is inactivated, give reactions comparable to the unheated filtrates containing active toxin. The possibility that heat-inactivated toxin derivatives might be responsible is ruled out by the pronounced "immediate" reactions produced by filtered solutions of the bacterial substance derived from washed, diphtheria bacilli, which although unheated contain no detectable traces of toxin. Further evidence is furnished by the "immediate" reaction given by our strain of xerosis bacilli, which although immunologically related to the diphtheria bacillus is totally devoid of the property of toxin production.

The preceding evidence seems to establish the described "immediate" skin reaction as a manifestation of true hypersensitiveness to diphtheria bacteria, of a type entirely distinct from that manifested by the common "delayed" reaction to the same bacteria. There are always objections to drawing analogies between bacterial hypersensitive phenomena in man and those in laboratory animals: in man the conditions of acquisition of the hypersensitiveness are almost always known and in other animals different methods of test are employed to detect the hypersensitive state. But, in spite of these limitations, the bacterial hypersensitiveness indicated by the "immediate" skin reaction of this man to diphtheria bacilli, like the so called bacterial "anaphylactic" condition of the guinea pig, appears to be an example of the same general type of hypersensitiveness as that obtained in a guinea pig systematically immunized with a chemically known antigenic protein; for the hypersensitive condition can be passively transferred, and the manifestation (or response) is produced by the injection of bacterial substances that are more heat-labile than those employed in the tuberculin test. On the other hand, the type of bacterial hypersensitiveness indicated by the "delayed" reaction of this man to diphtheria is like the bacterial hypersensitive condition responsible for the positive tuberculin test in a tuberculous guinea pig; for the condition of hypersensitiveness has not been passively transferred, and the manifestation (or response) is

produced by the injection of bacterial substances that are relatively heat-stable. Whether or not the above general analogy is justified, the experimental facts show that the "immediate" skin reaction of this individual involves an immunological system that is different from that involved in the production of his "delayed" reaction to the same bacteria. The injection of the entire diphtheria bacillus (or its culture filtrate) produces both reactions, but in all probability the bacterial constituent causing the "immediate" skin response is actually a different substance from the one causing the "delayed" reaction.

Many adults exhibit the "delayed skin reaction type" of hypersensitiveness to diphtheria bacilli but only one of those we have tested exhibits the "immediate skin reaction type." There is an equal lack of evidence that this man possesses greater tendency toward sensitization than that shown by others, or that he had greater opportunities to become sensitized to these particular bacteria. Although one always considers the individual's inherited tendency toward sensitization and his opportunities for contact with the antigen, all hypersensitive phenomena are influenced by a great number of such continually variable factors that one might conclude, as Cole (7) does for the causation of pneumonia, that either the acquisition or the manifestation of hypersensitiveness is usually an "accident." That is, the sensitization of this individual to diphtheria bacilli and the nonsensitization of others, like the production of pneumonia in one person and not in another, is probably due to no one factor alone but is more likely the result of a peculiar combination or coincidence of circumstances (for example, the presence of the particular bacillus at a time when certain tissues chanced to be unusually receptive).

Some authorities believe that the type of hypersensitiveness ("bacterial allergy") manifested by the tuberculin and other "delayed" skin reactions is important from the standpoint of resistance to the specific organism. But the "immediate" skin reaction represents a different immunological condition which has received little attention from the standpoint of its possible importance either in infection and resistance or in the manifestation of disease. It is not impossible that specific bacterial hypersensitiveness, while certainly not responsible for the definitely recognized symptoms of diphtheria, may account for

the occasional occurrence of the rashes[3] reported for diphtheria by observers who would not confuse them with the rashes of scarlet fever or of serum sickness.

The active immunization of people against diphtheria, especially with toxoid, introduces the question of the practical importance of the recognition of the "immediate skin reaction type" of hypersensitiveness to material derived from diphtheria organisms. This point will be discussed in a later paper but it should be noted here, that this type of hypersensitiveness is not being taken into account by those engaged in active immunization. The skin reaction now being used to detect individuals hypersensitive to the bacterial material of toxoid is the same "delayed" reaction as the well known "pseudo" of the Schick test; and the type of hypersensitiveness detected by it is not the same as that indicated by the "immediate" reaction described in this paper.

SUMMARY.

The paper describes an "immediate" skin reaction to derivatives of the diphtheria bacillus which is shown to be distinct from the "delayed" or "pseudoreaction" commonly seen in Schick tests on adults. The

[3] These rashes are transient, erythematous or urticarial in nature, and distinct not only from the lesions of actual diphtheritic infection of the skin but also from the purpura or ecchymotic lesions often seen in grave cases of the disease. They are of sufficient frequency to be included in the general reviews of diphtheria by Robinson (8), McCollom and Place (9), Gee (10), Jochmann (11), and others. The reports of their occurrence may be divided into three historical periods: (1) the early clinical observations, especially by American and English workers during the eighteenth century (12); (2) from the time of the clear definition of diphtheria in Bretonneau's classical papers to the time of the use of antitoxin; (3) the period of general use of antitoxin. The reports during the second of these periods are less complicated by the likelihood of confusion of the rashes of diphtheria with those of scarlet fever (13) or of serum sickness.

Jochmann (11) analyzed the question of these rashes and concluded that they occur in diphtheria uncomplicated by serum rash since they have been observed recently in cases not treated with antitoxin as well as in the period before the use of antitoxin. He interprets them as "toxische Erytheme, bedingt durch das Diphtherietoxin," but if the rashes were hypersensitive manifestations, the hypersensitiveness might not be toward the toxin but toward a non-toxic substance like the bacterial constituent involved in the hypersensitive individual studied in the present investigation.

"immediate" reaction was passively transferred to local areas of the skin of other people.

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EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. "Immediate" skin reaction to injection of the heated diphtheria filtrate employed as control in the Schick test; 15 minutes after injection.

FIG. 2. "Immediate" skin reactions to the Schick test; unheated filtrate injected in right arm and heated filtrate in left arm; 20 minutes after injection.

FIG. 3. Arms of the individual after the "immediate" reactions have disappeared and before the appearance of the "delayed" reactions; 3 hours after injection.

FIG. 4. "Delayed" reactions of this individual to the same injections showing a "combined" Schick reaction; larger reaction on right arm due to toxin in the unheated filtrate; 4 days after injection.

PLATE 3.

FIG. 5. Specificity of the "immediate" reaction in tests with heated suspensions of washed bacteria; injections as follows: (*a*) typhoid bacilli; (*b*) diphtheria bacilli; (*c*) colon bacilli; (*d*) salt solution; (*e*) xerosis bacilli; (*f*) *hofmanni* bacilli; (*g*) pneumococci; dose of other bacteria 2 times as great as diphtheria dose; 30 minutes after injection.

FIG. 6. Specificity of the "immediate" reaction in tests with broth culture filtrates; injections of filtrates as follows: (*a*) typhoid; (*b*) diphtheria; (*c*) colon; (*d*) broth; (*e*) xerosis; (*f*) *hofmanni;* (*g*) pneumococci; doses of other filtrates 5 times as large as diphtheria dose; 30 minutes after injection.

FIG. 7. "Delayed" reactions to injections described for Fig. 6; "delayed" reactions to typhoid and to colon filtrates had decreased at time of picture, but there was at no time a "delayed" reaction to xerosis; 24 hours after injection.

PLATE 4.

FIG. 8. Effect of previous heating treatment of diphtheria filtrate upon the "immediate" reaction; injections as follows: (*a*) filtrate heated 1 hour at 100°C.; (*b*) filtrate heated 30 minutes at 120°C.; (*c*) filtrate heated 20 minutes at 80°C.; (*d*) broth; 30 minutes after injection.

FIG. 9. Effect of previous heating treatment of diphtheria filtrate upon the "delayed" reactions to injections shown in Fig. 8.

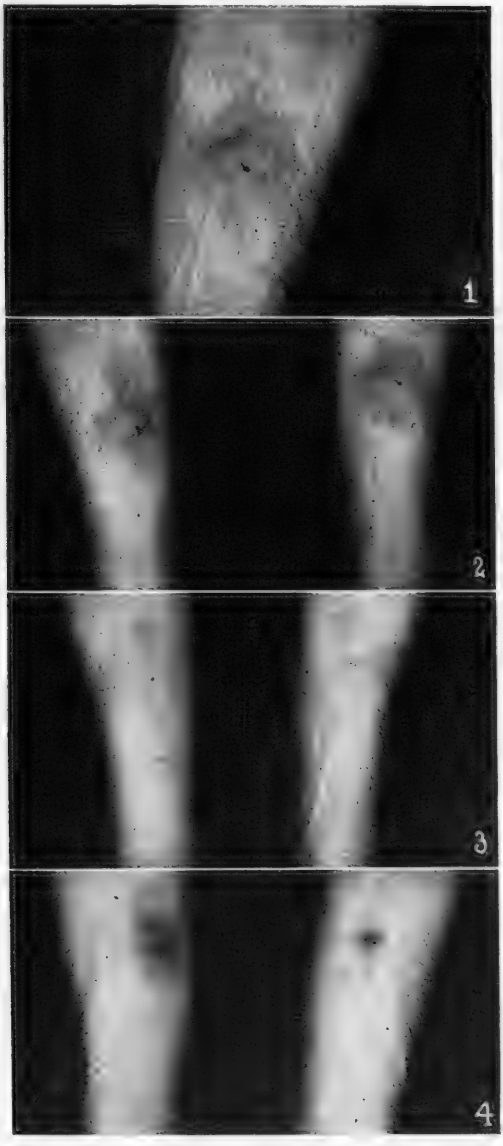
FIG. 10. Passive transfer of "immediate" skin reaction in tests with diphtheria filtrate; injections as follows: (*a*) diphtheria filtrate into area previously sensitized with hypersensitive individual's serum; (*b*) broth into normal skin area; (*c*) diphtheria filtrate into area previously injected with serum from another person; (*d*) diphtheria filtrate into area previously injected with salt solution; 30 minutes after injection.

PLATE 5.

FIG. 11. Passive transfer of "immediate" skin reaction in tests with heated washed diphtheria bacilli; skin areas prepared as described for Fig. 10, but heated bacteria used as test material instead of filtrate and salt solution control substituted for broth control in area (*b*).

FIG. 12. Specificity of passive transfer of "immediate" skin reaction; injections into previously sensitized skin areas, as follows: (*a*) typhoid filtrate; (*b*) diphtheria filtrate; (*c*) colon filtrate; (*d*) broth; (*e*) *hofmanni* filtrate; (*f*) xerosis filtrate; (*g*) pneumococcus filtrate; 30 minutes after injection.

FIG. 13. Effect of previous heating treatment of diphtheria filtrate upon the passive transfer of the "immediate" reaction; injections into previously sensitized skin areas as follows: (*a*) filtrate heated 1 hour at 100°C.; (*b*) filtrate heated 30 minutes at 120°C.; (*c*) filtrate heated 20 minutes at 80°C.; (*d*) broth; 30 minutes after injection. (Small triangular scar between areas (*a*) and (*b*) due to a burn.)



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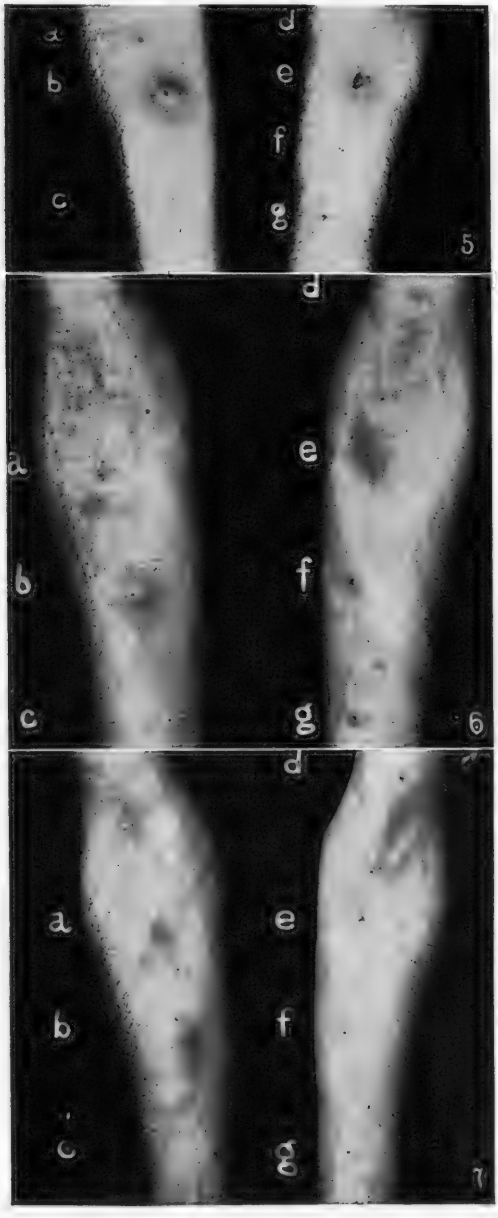
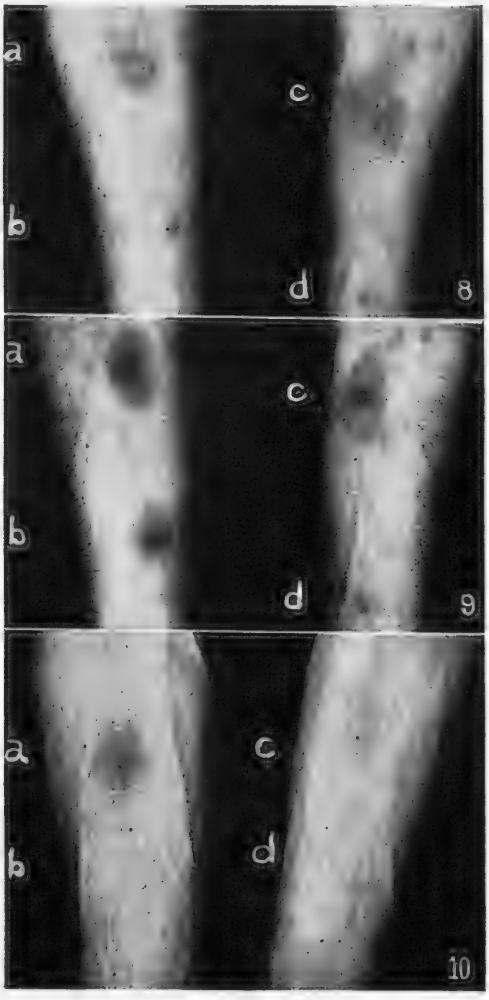


(Neill and Fleming: Hypersensitiveness to diphtheria bacilli. I.)



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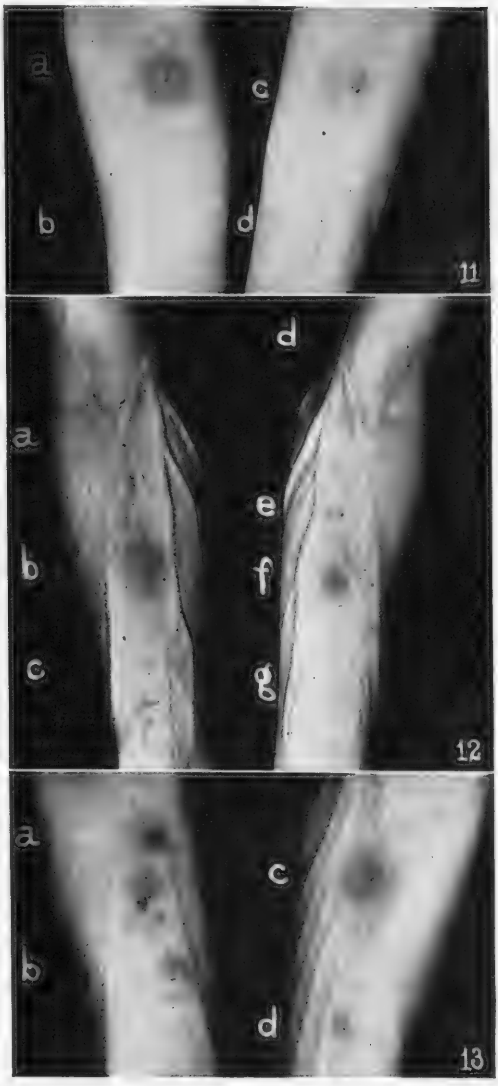

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THE GERMICIDAL AND HEMOLYTIC ACTION OF α-BROM SOAPS.

By ARNOLD H. EGGERTH, Ph.D.*

(*From the Department of Bacteriology, Hoagland Laboratory, Brooklyn.*)

(Received for publication, August 20, 1928.)

This paper is the first of a contemplated series of papers describing the germicidal action of various soap derivatives. The ordinary soaps are salts of fatty acids, the fatty acid radical consisting of a straight chain of carbon atoms to the first of which two oxygen atoms are attached, while the remainder are united to each other and to hydrogen. It is possible to replace one or more of these hydrogen atoms of the soap molecule by some other atom, such as a halogen, or a group of atoms, such as the hydroxy, amino, or other groups. If the substitution takes place at the carbon atom adjacent to the carboxyl group, the resulting substance is designated as an alpha substituted compound. As the parent unsubstituted fatty acids form an homologous series, the members of which differ from each other in the number of carbon atoms they contain, so the derivatives also form homologous series. By making a systematic study of several such series, it is hoped to throw some light on the relationship of chemical structure to germicidal action. The soaps are unusually favorable for such a study because they, more than any other germicidal substances we know, exhibit the interesting phenomenon of selective germicidal action (Walker, 1924).

In the present investigation, the alpha-brom derivatives of the ordinary saturated soaps have been prepared and tested. These are the simplest of the soap derivatives; they are also intermediate products in the synthesis of other series.

Preparation of the Alpha-Brom Fatty Acids.

Fatty acids are best brominated by the method of Hell (1881) by

* Van Cott Fellow, Department of Pathology, Hoagland Laboratory.

the addition of bromine and red phosphorus. The bromine first combines with the phosphorus forming PBr_5; this reacts with the fatty acids forming the acid bromides. An atom of bromine then replaces an atom of hydrogen, but only at the alpha position. The final product is an α-brom-acid bromide:

$$3\ RCH_2COOH + P + 11\ Br = 3\ RCHBrCOBr + HPO_3 + 5\ HBr$$

On pouring this reaction product into water, the acid bromide is converted to the fatty acid. Exact details for the preparation and purification of the various α-brom fatty acids are given by the following authors: α-bromobehenic acid, by Fileti (1907); α-bromoarachidic acid, by Baczewski (1896); α-bromostearic acid, by Hell and Sadomsky (1891); α-bromopalmitic acid, by Hell and Jordanoff (1891); α-bromomyristic acid, by Le Sueur (1905); α-bromolauric acid, by Auwers and Bernhardi (1891); α-bromocapric acid, by Bagard (1907). The preparation of α-bromocaprylic acid is not described in the literature; it is, however, easily prepared in the usual way and purified by distilling at 155°C. at a pressure of 10 mm. of mercury. α-Bromocaproic acid was purchased from the Eastman Kodak Co. A complete series of all the even numbered α-brom fatty acids from the behenic with 22 carbon atoms to caproic, with 6 carbon atoms, was thus obtained. Very careful purification of these acids is important, as partially purified products in several cases gave higher titers than the pure substances.

To form the corresponding soaps, the theoretical amount of KOH solution was added to weighed quantities of the fatty acids. The soaps were made up fresh for each experiment. Excess of alkali and high temperatures must be avoided, as hot alkali converts the brom to the hydroxy acids. However, the soaps of bromoarachidic and bromobehenic acids require a short boiling to bring them into solution.

The α-brom fatty acids and their soaps differ markedly from their parent unsubstituted fatty acids. The brom acids have a lower melting point and are stronger acids than the parent substances; their soaps are much more soluble in water and are far less hydrolyzed. For these reasons the α-brom soaps are much more satisfactory to work with than the unsubstituted ones. When the brominated soaps are added to acid buffer solutions, the brom fatty acids are liberated as fine and stable colloidal emulsions.

Technic of Germicidal Tests.

The potassium soaps were prepared, and serial dilutions made with sterile distilled water. Buffer solutions containing N/10 potassium phosphate and N/20 glycine were used to maintain the pH; the exact composition of these solutions is given in a previous paper (Eggerth, 1926). The sterile buffer fluids were inoculated with 24 hour broth cultures of the test organisms, and 0.5 cc. quantities were pipetted into series of small test-tubes; to these, 0.5 cc. quantities of the soap dilutions were added and the contents of the tubes well mixed. The tests were incubated at 37°C. and subcultured on glucose blood agar plates at the end of 30 minutes, 2 hours, and 18 hours.

The following test organisms were used:

1. *Streptococcus hæmolyticus*,—"Gay" strain.
2. *B. diphtheriæ*,—"Park-Williams No. 8" strain.
3. *B. typhosus*,—"Pfeiffer" strain.
4. *Staphylococcus aureus*,—old laboratory culture.
5. *Diplococcus pneumoniæ*, Type I,—recently isolated strain.
6. *Vibrio choleræ*,—American Type Culture Collection No. 528.
7. *Micrococcus ovalis*,—recently isolated strain.
8. *B. melitensis*,—old laboratory culture.
9. *B. lepisepticus*,—a virulent strain from Dr. Gay's laboratory.

The first four organisms are the same strains as used in previous investigations with soaps (Eggerth, 1926, 1927).

The inoculations in the germicidal tests were such that each cubic centimeter of final test fluid contained 0.02 cc. of a 24 hour broth culture, with the exception of *Staphylococcus aureus* and *B. typhosus*, where the inoculum was 0.01 cc.

Hemolytic tests were done by preparing a 2 per cent suspension of washed sheep red cells in 0.85 per cent NaCl solution containing N/20 potassium phosphate at pH 7.5. The soaps were diluted with salt solution, and 2 cc. of red cell suspension added to 2 cc. of soap dilution. The tubes were incubated at 37°C. and read.

DISCUSSION.

The effect of introducing a halogen atom into the molecule of an organic germicide is usually to increase germicidal action. This occurs

when the soaps are brominated (Figs. 1 to 4), the only exception being the bromocaproate, which is, in most cases, non-germicidal in a concentration of N/10. Also, when *B. typhosus* is the test organism, bromination does not increase toxicity (compare Table I with Fig. 4 of previous article, Eggerth, 1926). In most cases, however, germicidal action is considerably increased by bromination. Thus, potassium stearate has little or no action upon *Streptococcus* and *B. diphtheriæ* at pH. 7.5, whereas the corresponding brom derivative is highly active (Figs. 1 and 2). Potassium palmitate has no action upon *Staphylococcus aureus* at pH 7.5 in 2 hours, whereas the bromopalmitate is rapidly toxic in high dilutions (Fig. 3 and Table II). It is interesting to note that the bromostearate at pH 7.5 is five to ten times as germicidal as potassium oleate, a very soluble soap having the same number of carbon atoms (Eggerth, 1926).

Figs. 1 to 4 and Table I bring out a fact that is of great interest. For any given organism, germicidal action increases regularly as the number of carbon atoms of the soap increases, up to a certain point, after which it diminishes more or less rapidly. Thus, with *Diplococcus pneumoniæ*, the maximum is reached with α-bromostearate (Table I). With *Streptococcus hæmolyticus*, the bromopalmitate and bromostearate are about equally germicidal (Fig. 1), while with *B. diphtheriæ* (Fig. 2), *Staphylococcus aureus* (Fig. 3), and *Micrococcus ovalis* (Table I), the maximal titer occurs at the bromopalmitate. The bromomyristate is most germicidal for *B. lepisepticus*, and the bromolaurate for *V. choleræ* and *B. typhosus* (Table I). All of the Gram-negative organisms of the series tested have their highest titers with soaps of 12 or 14 carbon atoms, and the Gram-positive organisms with soaps of 16 or 18 carbon atoms. Higher titers are obtained with the Gram-positive than with the Gram-negative organisms.

The following hypothesis is suggested as an explanation of the above facts: The toxicity of the brom soaps for all species of bacteria increases rapidly with the length of the fatty acid chain. The ability to penetrate into the bacteria, however, diminishes with equal rapidity. Bacteria vary considerably in the permeability of their limiting membranes, the Gram-negative organisms being less permeable than the Gram-positive ones. The point of maximal germicidal titers, then, will be determined by the permeability of the organisms to the soaps.

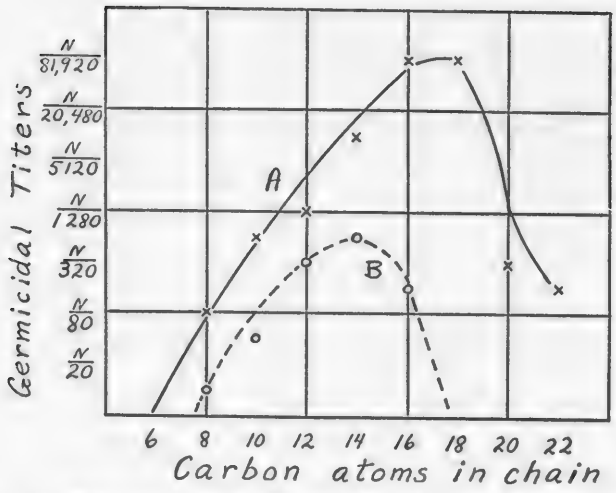

FIG. 1. The germicidal titers of α-brom soaps (*A*) and of unsubstituted saturated soaps (*B*) for *Streptococcus hæmolyticus*, at pH 7.5. The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.

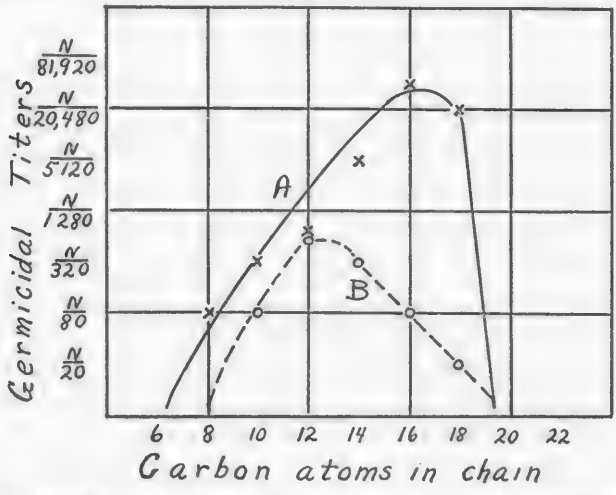

FIG. 2. The germicidal titers of α-brom soaps (*A*) and of unsubstituted saturated soaps (*B*) for *B. diphtheriæ*, at pH 7.5. The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.

TABLE I.

The Germicidal and Hemolytic Titers of α-Brom-Soaps.

	α-bromo-caproate	α-bromo-caprylate	α-bromo-caprate	α-bromo-laurate	α-bromo-myristate	α-bromo-palmitate	α-bromo-stearate	α-bromo-arachidate	α-bromo-behenate
No. of carbon atoms..	6	8	10	12	14	16	18	20	22
				Diplococcus pneumoniæ					
pH									
6.5		N/640	N/5120	N/20,480	N/163,840	N/327,680	N/327,680	N/1280	N/1280
7.5	0	N/160	N/1280	N/5120	N/40,960	N/81,920	N/163,840	N/640	N/640
8.5		N/80	N/640	N/2560	N/40,960	N/40,960	N/81,920	N/640	N/640
				Streptococcus hæmolyticus					
6.0		N/320	N/2560	N/5120	N/40,960	N/163,840	N/163,840	N/640	N/160
7.5	0	N/80	N/640	N/1280	N/10,240	N/81,920	N/81,920	N/320	N/160
8.5		N/40	N/320	N/640	N/5120	N/40,960	N/40,960	N/160	N/80
				Staphylococcus aureus					
6.0		N/320	N/1280	N/2560	N/20,480	N/81,920	N/320	0	0
7.5	0	N/80	N/320	N/640	N/5120	N/40,960	N/320	0	0
8.5		N/40	N/320	N/640	N/2560	N/20,480	N/80	0	0
				B. diphtheriæ					
6.0		N/320	N/1280	N/2560	N/20,480	N/81,920	N/40,960	0	0
7.5	0	N/80	N/320	N/640	N/5120	N/40,960	N/20,480	0	0
8.5		N/40	N/160	N/320	N/2560	N/20,480	N/10,240	0	0
				Micrococcus ovalis					
6.0		N/320	N/640	N/1280	N/10,240	N/20,480	N/10,240	0	0
7.5	0	N/80	N/160	N/320	N/2560	N/10,240	N/5120	0	0
8.5		N/40	N/80	N/160	N/1280	N/5120	N/2560	0	0
				Vibrio choleræ					
6.0		N/320	N/1280	N/10,240	N/10,240	N/5120	N/320	0	0
7.5	N/20	N/80	N/320	N/2560	N/1280	N/640	N/80	0	0
8.5		N/40	N/160	N/1280	N/640	N/320	N/80	0	0

Where permeability is low, so that only the lower soaps can act, the maximal titers will be low; where permeability is high and the longer chained soaps can act, the maximal titers will be high.

TABLE I—*Concluded.*

	α-bromo-caproate	α-bromo-caprylate	α-bromo-caprate	α-bromo-laurate	α-bromo-myristate	α-bromo-palmitate	α-bromo-stearate	α-bromo-arachidate	α-bromo-behenate
No. of carbon atoms..	6	8	10	12	14	16	18	20	22
B. lepisepticus									
6.0		N/160	N/2560	N/5120	N/10,240	N/1280	N/160	0	0
7.5	0	N/80	N/320	N/2560	N/5120	N/640	N/80	0	0
8.5		N/40	N/160	N/1280	N/2560	N/320	N/80	0	0
B. melitensis									
pH									
6.0		N/320	N/640	N/5120	N/10,240	N/10,240	0	0	0
7.5	N/10	N/80	N/320	N/2560	N/2560	N/2560	0	0	0
8.5		N/40	N/160	N/1280	N/2560	N/1280	0	0	0
B. typhosus									
6.0		N/40	N/80	N/80	0	0	0	0	0
7.5	0	N/20	N/40	N/80	N/20	0	0	0	0
8.5		N/20	N/40	N/80	N/20	0	0	0	0
Hemolytic titer for sheep red cells									
7.5	0	N/160	N/320	N/2560	N/5120	N/20,960	N/20,960	N/640	N/640

The lowest dilutions tested were N/10 for the bromocaproate, N/40 for the bromoarachidate and bromobehenate; all others, N/20. Time of germicidal tests, 2 hours; time of hemolytic tests, 30 minutes. Temperature, 37°C.

It was observed that when the titer of a soap for an organism fell on the ascending limb of the curve (as in Figs. 1 to 4), such a titer was very constant in different experiments, never in any case varying by more than one tube in the series. But when the titers fell on the descending limb of the curve, the results in different experiments were extremely variable; those given in the figures and tables represent the mean of several observations. Thus, the bromoarachidate gave, with *Streptococcus*, at pH 7.5 for 2 hours, a titer of N/2560 in one experiment, N/640 in a second, N/160 in two experiments, N/80 in a fifth. The bromostearate gave with *Staphylococcus* the following titers in successive experiments: N/40, N/2560, N/320, N/640, N/80, N/640. The

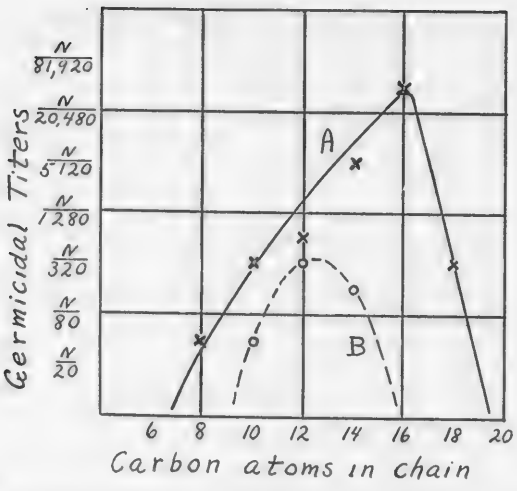

FIG. 3. The germicidal titers of α-brom soaps (*A*) and of unsubstituted saturated soaps (*B*) for *Staphylococcus aureus*, at pH 7.5. The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.

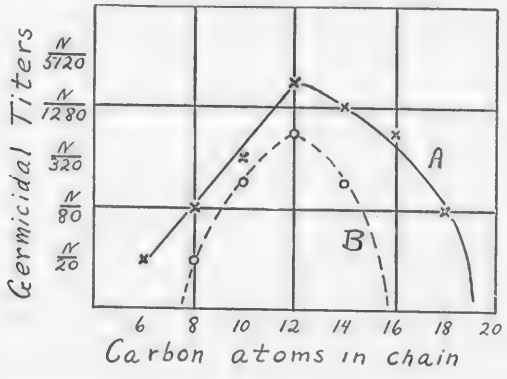

FIG. 4. The germicidal titers of α-brom soaps (*A*) and of unsubstituted saturated soaps (*B*) for *Vibrio choleræ*, at pH 7.5. The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.

same thing occurred in hemolytic tests, the titers of the bromoarachidate and bromobehenate varying between N/80 and N/1260, while with the other soaps the hemolytic titer was very constant in different experiments.

In general, the brom soaps are very rapid germicides. In most cases, the 30 minute titer was found to be one-half the 2 hour titer. Shorter periods than 30 minutes were tried with two organisms, *Staphylococcus* and *Streptococcus;* the results are given in Table II. The extremely rapid action of high dilutions of the bromopalmitate against these two pyogenic organisms is noteworthy; a concentration of N/2560 being

TABLE II.

Germicidal Titers of Potassium α-Bromopalmitate at pH 7.5 for Different Time Intervals.

Test organism.....	*Staphylococcus aureus*	*Streptococcus hæmolyticus*
Time		
15 sec.	N/1280	N/2560
30 sec.	N/2560	N/2560
1 min.	N/5120	N/5120
5 min.	N/5120	N/5120
10 min.	N/10,240	N/10,240
30 min.	N/20,480	N/40,960
2 hrs.	N/40,960	N/81,920
18 hrs.	N/40,960	N/163,840

Temperature, 37°C.

about 1 part by weight to 7500 of water. Two other recently isolated strains of *Staphylococcuss aureus* gave identical results with this soap.

As can be seen in Table I, the effect of the pH on the germicidal action of the brominated soaps is similar to that of the parent unsubstituted soaps; if anything, the results are more clear-cut. It was found in a previous paper (Eggerth, 1926) that the lower members of the saturated series of soaps were most germicidal in acid reactions, while the longer chained soaps were most germicidal at an alkaline pH. It was suggested that this difference was due to the high insolubility of the higher fatty acids liberated at the acid pH. The brom fatty acids, however, are more soluble, hence, with certain organisms such as *Diplococcus pneumoniæ* and *Streptococcus hæmolyticus*, the entire series of bromi-

nated soaps including the behenic with 22 carbon atoms is more active in acid than in neutral or alkaline reactions. The data obtained in this investigation, therefore, confirm and strengthen the conclusion previously arrived at: that whenever they are sufficiently soluble to be tested, the fatty acids are more germicidal than the corresponding soaps.

The hemolytic titers of the α-brom soaps are given in Table I; the results are in every way similar to those obtained in the germicidal tests.

SUMMARY AND CONCLUSIONS.

1. The soaps of the α-brom fatty acids are usually more germicidal than the unsubstituted soaps. Only when *B. typhosus* was the test organism was there no increase in germicidal action.

2. For any test organism, germicidal action of the brominated soaps increases rapidly with increasing molecular weight up to a certain point, then diminishes. This is likewise true of the hemolytic titer. The point of maximal germicidal action varies with the different species of tests organisms. In the series studied, brominated soaps of 12 and 14 carbon atoms were most germicidal for the Gram-negative organisms, while soaps of 16 and 18 carbon atoms were most germicidal for Gram-positive organisms.

3. The brominated soaps are, in general, more active in acid that in neutral or alkaline reactions. The reasons for this have been discussed in a previous paper, in which a similar phenomenon with unsubstituted soaps was observed.

4. For certain organisms, the brominated soaps are among the most rapid and potent germicides known.

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STUDIES ON FOWL PARALYSIS (NEUROLYMPHOMATOSIS GALLINARUM).*

I. Clinical Features and Pathology.

By ALWIN M. PAPPENHEIMER, M.D., LESLIE C. DUNN, Ph.D., and VERNON CONE, M.D.

(*From the Storrs Agricultural Experiment Station, Storrs, and the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.*)

Plates 6 to 12.

(Received for publication, August 23, 1928.)

The disease known to poultrymen as fowl paralysis or range paralysis is one which, in spite of its economic importance and great biological interest, has been little studied.

The first account of the disease to which we have found reference is that of Marek (1) in 1907. In four roosters, three of them Orpingtons, and all from the same farm, there developed during the winter months, symptoms of paresis of the legs and wings, unequal on the two sides, and accompanied by more or less muscular atrophy. The diet was adequate, comprising potatoes, barley, bran, clover and mixed grains, and the chickens were allowed free range. Two of the birds made a partial recovery, one died after 25 days, one was killed 5 weeks after the onset of symptoms, and the description of the pathology is based upon the careful study of this bird.

Grossly, there was noted marked thickening of the right lumbar plexus (the side on which the paralysis was more marked). Most of the spinal nerve roots, especially on the right side, were also thicker than normal.

Microscopic examination was made of four segments of the cervicodorsal and lumbosacral regions of the cord with their nerve roots, of the lumbar plexus, sciatic and femoral nerves and the extensor quadriceps femoris muscle. The tissue was

* Preliminary reports of this work have been presented at the November meeting of the New York Pathological Society, 1925; at the Cleveland meeting of the American Society of Experimental Pathology, December 28, 1925; and at the World Poultry Congress in Ottawa, August 2, 1927. Much of the material in this and the following paper has been included in Bulletin 143 of the Storrs Agricultural Experiment Station, 1926.

fixed in Mueller's fluid, stained with Weigert-Wolters and counterstained with alum-cochineal and Van Gieson.

In the plexus and sciatic nerves, there was almost a complete loss of nerve fibers. There was a dense and uniform infiltration of mononuclears, in places aggregated into dense clumps. The perineurium was but slightly thickened and contained only scattered cells. Nests of mononuclears were present in the surrounding connective tissue, chiefly in the vicinity of small blood vessels.

The spinal cord and the nerve roots, especially upon the right side, were also infiltrated. In the cord, the cellular infiltrations were almost limited to the white matter, but in one segment, there was a perivascular accumulation in the central grey.

The degeneration of the nerve fibers was regarded by Marek as secondary to the cellular invasion. The cause of the polyneuritis could not be determined, but the disease was obviously not identical with the neuritis described by Eijkman (2) in chickens fed on polished rice. In the latter condition, death ensued within 10 days, and the changes in the nerves were purely degenerative.

Kaupp (3) in 1921 reported rather fully upon observations first made in 1914. In this paper, reference is made to outbreaks occurring in North Carolina, Maryland, Virginia, New Hampshire, New Jersey, Connecticut and Massachusetts. No information could be obtained by him as to the occurrence of a similar disease in foreign countries.

The clinical features as described by Kaupp are lameness in one or both legs, drooping of wings, partial blindness, finally diarrhea, complete paralysis and death days or weeks after the onset. Recovery, in Kaupp's experience, never occurred. All breeds were susceptible.

As regards the lesions, Kaupp found no changes in the cerebellum, medulla, crura or brachial plexus. In the cord, however, corresponding segmentally to the paralysed part, there was congestion of peripheral veins, round cell infiltration in both ganglionic and non-ganglionic portions of the cord, chiefly perivascular. There was degeneration of the ganglion cells, atrophy of the fibers and at times a liquefaction necrosis. The process was interpreted as a transverse myelitis. Kaupp studied the disease experimentally, but without arriving at any definite results. Repeated efforts to isolate a causative organism were without success. Inoculation of heart blood, liver, kidneys, spleen, various parts of brain and cord, especially of the affected segments, into rabbits, guinea pigs, young and year old fowls all failed to reproduce the disease. In spite of these negative experiments, Kaupp regarded the disease as infectious.

An interesting paper treating of an apparently identical disease occurring in the Netherlands, is that of Van der Walle and Winkler-Junius (4). The authors studied an epizootic which occurred in the months of October and November, 1921. A large number of fowl were affected with paralysis, which ended acutely after a few days or weeks. Most of the affected birds died, but the appetite and general appearance of the chickens was unchanged even in the last stages of the

disease. Avitaminosis could be excluded, and the food contained neither arsenic, copper, lead nor zinc.

Two of the cases were examined pathologically. Inflammatory infiltrations of polynuclear and mononuclear leucocytes were found in the swollen dorsal ganglia; the meninges and spinal cord were likewise infiltrated, and in one of the birds, the sacral and lumbar plexus showed mononuclear infiltration.

The alterations of the nerve elements were regarded as secondary to the infiltrations, and the apparently inflammatory nature of the lesions suggested the term "neuromyelitis gallinarum" for the disease. No mention is made of lesions of the brain.

A number of experiments to demonstrate the infectious nature of the disease led the authors to conclude that this epizootic was due to a filtrable virus. We shall discuss these in connection with our own experiments on transmission of the disease.

May, Tittsler and Goodner (5) in December, 1925, issued a preliminary report of field observations and laboratory findings in paralysis of the domestic fowl. The lesions are very briefly and incompletely described. The authors state that microscopic examination of brain and spinal cord has given little information in regard to the seat of the disease. Sections of brain and cord confirmed Kaupp's findings, but the lesions are not described in detail, and no mention is made of alterations in the peripheral nerves. Both aerobic and anaerobic cultures from the tissues of infected birds consistently failed to show any infective organisms. Attempts to transmit the disease by association and contact, by feeding material from paralysed birds or by inoculation of blood, emulsified brain or spinal cord, intravenously, intraperitoneally or intraspinally were all negative. None of the inoculated animals developed paralysis during an observation period of 2 months.

The authors discuss the possible rôle of intestinal parasites and reach the conclusion that there is no relation, or at least that the presence of intestinal worms or coccidia does not necessarily produce paralysis.

The most recent paper on the subject is that of Doyle (6) from the Indiana Agricultural Experiment Station. Both the symptoms and the pathology of the disease are very completely described. The disease occurs in birds from 4 to 8 months old, and is more prevalent in summer and fall. The symptoms depend upon the portion of nervous system involved. The occurrence of encephalitis is responsible for the lethargic symptoms frequently noted. Iritis, when present, causes a contracted, non-responsive pupil. There is a good description of the histopathology of the brain, cord and peripheral nerves.

Efforts to demonstrate the infectious nature of the disease were fruitless; the author's experiments will be considered in detail in the second paper, but it is interesting to note here that in spite of his failure to establish the transmissibility of the disease, he is still convinced that it is due to an infectious agent.

Clinical Features of the Disease.

The first appearance of the disease is usually signalized by drooping of a wing on one side, a lack of coordination in walking, followed by a lame or limping gait, and ending in complete prostration. The limbs are not symmetrically affected, even in advanced cases. The chickens usually lie upon the side showing the greater paralysis, and the impairment of mobility is at first unilateral, or at least more marked upon one side than the other.

In the earlier stages of the disease, there is little muscular atrophy, but after the chicken has been helpless for a longer period, the wasting may become extreme. Whether the atrophy is due to the affection of the nerves, to the inactivity or to the inability of the chicken to procure food has not been determined.

The paralysis is more often spastic than flaccid, and sometimes accompanied by increased reflexes or even by clonic spasms. The paralysis is only exceptionally complete, and even in birds which lie immobile and helpless, the struggles during anesthetization are attended by vigorous movements of flexion or extension. Reflex movements and muscular reaction to mechanical nerve stimulation have been noted in the paralysed limbs after death. The curious postures assumed by the paralysed birds are illustrated by Figs. 1 and 2.

The duration of the disease, as judged from the onset of the paralytic symptoms to the termination in death or rarely in recovery, is very variable. With proper care, life may be prolonged indefinitely, and the nutrition and general condition is often surprisingly good, even after months of complete helplessness when the birds are kept in confinement with access to food and water. Thus one of ten early cases in the station flock at Storrs became prostrated early in October, 1922. With careful feeding in an individual coop, it lived until January 1, 1923. Recovery has been noted in several instances, and we have gained the impression that there frequently occur periods of transient improvement. On the other hand, death may suddenly occur with few premonitory symptoms, the diagnosis being revealed only by the pathological study of the cases.

The onset of the paralysis may be very sudden. We have observed one case in which complete loss of power in the legs developed in less than 3 hours.

Partial or complete blindness in one or both eyes, accompanied by a change in the color of the iris from yellow-brown to slate-grey, was observed in several paralysed chickens, and in two non-paralysed birds from the same flock. The alterations which underlie this condition will be described below.

As regards the age incidence, it would appear that young birds are most frequently affected. In our experience, the earliest symptoms in a spontaneous case were observed at 12 weeks, and the oldest case in our records developed paralysis at 15 months, 18 days. In the experimental material, the earliest clinical case was observed at 10 weeks of age. As will become apparent later, the characteristic lesions do not always reveal themselves in typical paralytic symptoms. We shall show also that mild cases exist in which the diagnosis cannot be made from symptoms alone.

Epidemiology and Distribution.

The records of the Storrs Experiment Station flock of White Leghorns in which each year about 1500 growing chicks and 800 adult chickens come under observation, have yielded data of some value in regard to the natural history of the disease, and instructive information has been obtained from visits to affected farm flocks. It is impossible to present an analysis of these data within the confines of this present article, but we may refer briefly to some of the more interesting deductions.

(1) The disease appears to be epidemic and to reappear year after year on the same farm. In cases where its origin has been observed, it has appeared suddenly and has thereafter persisted and become endemic in certain foci. Efforts to prevent its recurrence by rearing new flocks on new ground on the same farm, have, in several cases, shown no significant effect on the persistence of the disease. (2) Paralysis occurs in both sexes and in all of the chief breeds of fowls. There is some indication of breed differences in susceptibility to the disease, although the observations on which these are based are in no sense experimentally controlled and the difference may have been due to other conditions. (3) The disease first appears usually in growing chickens from 3 to 6 months of age, and new cases continue to de-

velop throughout the first 18 months of life. It is probably rare in older fowls although a few clinical cases have been observed. (4) In flocks in which an outbreak has occurred, paralysis usually causes an appreciable mortality each year thereafter. Mortality may range from a few per cent, up to 60 per cent and over.

Pathology.

The following description is based upon a study of 60 spontaneous cases in all stages of the disease. Many more have been autopsied in the course of the experimental work. The chickens were obtained from a number of different sources, and characteristic lesions have been found in material from Maine, New Hampshire, Massachusetts, Pennsylvania, New Mexico, California and Ontario. The following breeds are represented: White Leghorns, Silkies, Silver-Spangled Hamburgs, Plymouth Rocks, Buff and White Wyandottes and Rhode Island Reds. In this series, the lesions were identical, irrespective of breed.

Numerous chickens presenting no clinical evidence of paralysis, have been studied.

The brain after fixation in Zenker's was cut in serial blocks so as to include sections of cerebrum, optic lobes, cerebellum, pons and medulla. The entire spinal cord was removed, and longitudinal sections examined at all levels. The dorsal root ganglia, and the brachial plexus and sciatic nerves were also taken for study, and routine sections from all the principal viscera. Zenker's fluid, and 10 per cent formol or Bouin's fluid in special cases, were used as fixatives. In addition to routine histological stains, sections from individual cases were studied by the Marchi and Pal-Weigert methods, and a variety of staining methods were used in the attempt to demonstrate microorganisms in the tissue.

Gross Lesions.

The general nutrition of the birds varied, depending upon the duration of the disease and the care which the chickens had received. Many typical examples showed excellent nutrition; others extreme emaciation. Often the feathers about the cloaca were matted with excrement, owing to a terminal diarrhea or incontinence.

No recognizable alterations were found in the brain, or its covering. The posterior root ganglia of the cord were found enlarged, and in the majority of cases, to such a degree that, after a little experience, the histological changes could be

predicted with fair certainty. Not only were the ganglia enlarged, but they appeared abnormally translucent, and usually they had a slightly yellowish tinge in comparison with the cream-white of the cord itself. On close inspection, one could frequently discern a translucent strip extending into the contiguous tissue of the cord. The enlargement of the ganglia, in most cases, was not symmetrical (Fig. 3).

In one case there was found a translucent, tumor-like enlargement of the acoustic nerve. In another, one of the trigeminal nerves was thickened. We have made no systematic study of the cranial nerves.

No striking gross changes were found in the spinal cord itself. The *peripheral nerves*, in particular those of the brachial plexus and the sciatic nerves, were frequently thickened and the same smoothness, translucency and yellowish discoloration were noted as in the posterior root ganglia (Fig. 3). Small hemorrhages were occasionally seen.

Lymphomatous tumor-like masses were discovered in six birds of this series, or about 10 per cent. These appeared to originate in the ovary, but growths were seen in the liver, kidneys, lungs, adrenals and muscle, and in one instance, the spinal cord was infiltrated with the same type of tissue. The finer structure of these lymphomatous masses and their possible relation to the lesions of the nervous system, will be discussed below.

None of the lesions occasionally met with, such as edema and atelectasis of the lung, suppurative bronchiectasis, ulceration of the intestine associated with severe coccidiosis, etc., seemed to be related to the disease under consideration. *Heterakis papillosa* was present in the ceca in variable numbers in practically every case, *Heterakis perspicillum* in several and cestodes occasionally.

Microscopic Lesions.

Striking alterations were found in the brain, spinal cord, dorsal root ganglia, spinal nerve roots and peripheral nerves. The distribution and intensity of the lesions varied from case to case, but in general the lesions resembled one another sufficiently to establish a definite pathological entity.

Peripheral Nerves.

The most severe lesions were seen in the peripheral nerves and nerve roots. They consisted essentially of an infiltration of smaller and larger mononuclear cells between the nerve fibers, separating the individual fibers and accompanied by more or less edema. This infiltration was at times so massive as to replace almost completely the nerve tissue. More commonly, the cells were loosely distributed and in some areas, very sparsely. It was rare for all the bundles of the nerve to be affected, and never did the infiltration extend uniformly throughout the entire length of the section (Figs. 4 to 6).

Degenerative changes in the nerve fibers varied greatly in the different cases examined, and were roughly correlated with the extent and intensity of the cellular infiltration. In many instances, where the lymphoid invasion was sparse and scattering, the degeneration of the nerve fibers was not detectable with the usual stains. At the other extreme, many of the fibers had disappeared, their places being taken by a vacuolar or foamy tissue, often filled with coagulated edema fluid, and containing free fat globules and numerous fat-laden phagocytes, derived in part from the swollen sheath cells. The myelin sheaths appeared collapsed, and Pal-Weigert preparations gave evidence of advanced degeneration (Figs. 7, 8). In some sections, there could be noted a proliferation of the spindle-shaped sheath cells, amongst which mitotic figures were abundant.

Even when these degenerative changes were well advanced, the persistence of the neuraxones in the infiltrated nerves could be demonstrated in silver carbonate preparations (method of Rio del Hortega), and it is probably due to this fact that paralysis, even in advanced cases, is rarely complete.

The smaller forms of infiltrating cells appear to be morphologically identical with lymphocytes. In the nodular accumulations which crowd aside the adjacent nerve fibers, the center of the mass is composed of somewhat larger elements, with more vesicular nuclei, and here mitoses are frequent. In addition to cells of the lymphocytic type, which predominate, there are numerous cells with more abundant basophilic cytoplasm, peripherally disposed chromatin and a juxtanuclear clear space, which closely resemble mammalian plasma cells. These are vividly brought out in sections stained with Pappenheim's methyl green pyronine. There are also large mononuclear cells with vacuolar cytoplasm corresponding to the fat-laden phagocytes derived from the cells of Schwann's sheath. In paralysed chickens stained intravitally with trypan blue, many of the large mononuclears in the infiltrated nerves, particularly in the vicinity of the blood vessels, were found to accept the dye though less intensely than the Kupffer cells, or the clasmotocytes of the spleen and other tissues.

In a case recently studied, most intense lymphoid infiltrations were present in the macroscopically thickened vagus and mesenteric nerves.

Spinal Cord.

In the spinal cord, in addition to the regional infiltrations accompanying the entrance of the nerve roots, there are found focal accumulations of wandering cells most commonly in the white matter, but in some instances also in the central grey. Lesions of this type, which may be termed submiliary nodules, seem to be limited to the vicinity of small blood vessels. The cells composing them are in part definitely lymphoid in type, in part, cells with paler, less spherical and somewhat larger nuclei, the origin of which could not be accurately determined. They are most probably mobile glial elements. A very constant feature of these nodules is the presence of spherical, deeply stained chromatin particles of varying size and shape interspersed amongst the intact nuclei. These appear to be derived

by a massing of the chromatin against the nuclear membrane and its subsequent extrusion; often they are surrounded by a narrow clear zone.

In no case was there definite necrosis in the center of the nodules, nor have we observed the occurrence of giant cells.

Degenerative changes in the ganglion cells of the cord were seen only where the lymphomatous infiltration was so massive as to bring about extensive destruction of the cord tissue. The most obvious type of degenerative change was a hyalinization of the cytoplasm, with chromatolysis, and karyolysis.

Posterior Root Ganglia.

The posterior root ganglia, as one might expect from the alterations in the gross appearance, frequently show an intense infiltration with mononuclear cells (Figs. 10, 11). This was sometimes diffuse, sometimes rather circumscribed. The extent and intensity of the infiltrations varied greatly in different ganglia from the same case. The ganglion cells were usually intact, even when the lymphoid infiltrations were extreme.

Brain.

The majority of the birds showed lesions in the cerebrum, cerebellum, optic thalamus and pons. These were always exquisitely focal in distribution. The massive and diffuse infiltrations seen in the nerves and spinal cord were never found in the brain. The lesions found were of the two types; either compact perivascular rings of small densely staining lymphoid cells, or submiliary nodules composed of lymphoid cells and paler elements, like those described as occurring in the white matter of the cord. In two cases, the lesions seen in the molecular layer of the cerebellum had a slightly different character. They were very sharply circumscribed, and composed almost exclusively of pale epithelioid elements showing a more or less definite radial arrangement. There were very few lymphoid cells in the nodules, which gave the impression of healing lesions.

The foci of cellular infiltration in the cerebrum were often very scarce, and in a few of the birds, even careful study of a number of sections at different levels failed to disclose them.

Although perivascular infiltration of the meningeal vessels was repeatedly seen, in only one case were there observed a more diffuse infiltration and thickening, accompanied by the presence of foamy, fat-containing cells.

Sympathetic Nervous System.

A systematic study of the sympathetic nervous system was not undertaken, but sympathetic ganglia and nerves were frequently included in the visceral sections, particularly in the section of adrenal (Fig. 12). In a number of cases, mononuclear infiltrations more or less intense were present in the ganglia and nerve trunks, so that it may be said with certainty that the disease involves the

sympathetic system. To what extent this affects the clinical picture of the disease, can only be surmised.

Thickening and cellular infiltrations have also been repeatedly found in the vagus nerves and in the nerve trunks accompanying the mesenteric vessels.

Eye Lesions.

In three birds showing partial or complete blindness, there was observed in the living animals a change in the color of the eye, from a yellow-brown to a bluish grey. This was readily explained by the microscopic changes. The iris was found to be thickened, edematous and infiltrated with wandering cells. These were of two distinct types—small, deeply staining lymphoid cells with little cytoplasm, and larger mononuclears resembling mammalian plasma cells, the nucleus being eccentric, and the cytoplasm strongly basophilic. These larger elements were aggregated into compact clusters, situated especially near the anterior surface. Occasional eosinophilic polymorphonulcears were also seen. There was slight edema, and a granular coagulum, and in some sections mononuclear cells of various types were present in the anterior chamber (Fig. 13).

The cornea was normal in structure. The retina was unaltered and the optic nerve heads showed only a minimal infiltration. Groups of lymphoid cells, however, were present in the conjunctiva and in the extraocular muscles.

Visceral Lesions.

Lungs.—No lesions were found which could be correlated with the cellular infiltrations present in the central nervous system. The only exception to this statement is in the case of chickens in which there were present lymphomatous masses in the abdominal viscera. In several of these, the lungs were also the seat of lymphomatous growths in the form of diffuse infiltrations in the walls of the bronchi and perivascular areolar tissue. In general, however, the lymphoid tissue which is normally found in the mucosa of the larger bronchi, was not hyperplastic.

Myocardium.—In the routine study of sections both from paralysed and normal chickens, one finds almost without exception, focal aggregations of wandering cells amongst the muscle fibers or in the loose connective tissue about the larger blood vessels. Sometimes these are composed entirely of lymphoid or larger mononuclear cells, with basophilic cytoplasm; at times there is a predominance of eosinophilic polymorphonuclears or of larger mononuclears with coarse eosinophilic granules—presumably, myelocytes. So frequently are these small cell aggregations to be found that it seems difficult to regard them as the expression of an infectious lesion, and still more difficult to believe that they are in any way related to the disease under consideration. It seems more probable that they are merely extramedullary leuco- and lymphopoietic foci.

On the other hand, we have encountered instances in which the myocardial infiltrations are so intense and widespread that they cannot be interpreted merely

as areas of heterotopic blood formation. Two types of changes have been seen. The first is a simple accumulation of small round cells crowding apart the intact muscle fibers, mantling the small arterioles and at times forming large oval aggregations which completely replace the muscle tissue over a considerable area.

The other type of lesion is more definitely inflammatory, and has been seen but once. It occurred in a Barred Plymouth Rock, which presented somewhat atypical lesions. There was iritis with blindness. The brain and cord were the seat of intense lesions of the usual character. The nerve trunks were not involved, but there was extensive perineuritis. A cross-section of the ventricle showed that a large part of the myocardial tissue was replaced by granulomatous areas containing a variety of cell forms—fibroblasts, giant cells, often surrounding remains of necrotic muscle fibers, lymphocytes, many of them fragmented and pycnotic, and rare eosinophilic polymorphonuclears. Although the individual muscle fibers in the affected areas were degenerated or necrotic, there were no larger areas of coagulative necrosis, nor tubercle-like lesions. The pericardium was invaded by the granulomatous tissue, leaving the overlying serous cells intact. It is of course possible that in this case the myocardial lesions, which are frankly inflammatory in type, may have been due to some other agent than that causing the lesions in the iris and central nervous system. It seems possible, however, that we are dealing with an unusual and atypical reaction to the same agent.

Liver.—In this organ, also, it is difficult to draw a sharp line between the normal and the pathological. Infiltrations of the periportal connective tissue with lymphoid cells, and not infrequently with eosinophilic, mono- and polymorphonuclear leucocytes, are present in practically every section examined, whether of normal or paralysed fowl. One finds also small, compact spherical masses of small mononuclears in any portion of the liver lobule, and these appear to be a normal feature of the chicken liver.

In certain of the paralysis cases, these infiltrations have become so massive as to leave no doubt as to their pathological nature (Fig. 14). They may even reveal themselves macroscopically as greyish areas obliterating the normal architecture of the liver lobule, and in several of the birds with large lymphomatous masses in the region of the ovary, the liver lesions were of a distinctly nodular character, grossly and microscopically suggesting a neoplasm. It would seem, therefore, that the same agent which is responsible for the infiltrations of the nervous system may at times incite the lymphoid tissue of the liver to abnormal and excessive growth.

Pancreas.—Interstitial lymphoid masses were present in a few cases. There were no lesions of the parenchyma.

Spleen.—We have found neither gross nor microscopic lesions.

Kidney.—The situation here is analogous to that in the liver. In every section of kidney, lymphoid infiltrations of variable extent may be found between the tubules. In certain cases, these may be so extreme as to separate widely or actually replace the parenchymal elements. In several of the birds with large

tumors arising about the ovary, there was direct extension into the kidney substance.

Adrenal.—Here again in routine sections, one finds very frequently compact aggregations of lymphoid cells in any portion of the gland, and such lymphoid foci, as in the adult human adrenal, may be looked upon as a normal histological feature of the gland. In many of the paralysed fowls, these infiltrations are very extreme. They appear to take their origin in the sympathetic ganglia and nerve trunks, which, in the chicken adrenal, are intimately incorporated with the glandular elements. We have already referred to the periadrenal nerves as very frequently and characteristically involved.

Ovary.—In the majority of the typical cases the ovary showed no pathological changes. In a certain proportion of the cases, this organ, as has been stated, gave origin to large lymphomatous masses of an invasive character.

Testes.—No infiltrations have been found in the testes of normal or paralytic fowl, and the male gonads seem to be, of all the viscera studied, the least favorable site for a localization of the lesions.

Our material does not provide a certain answer to the question as to whether the presence of the disease arrests or suppresses spermatogenesis. In the series of 60 spontaneous cases, there were but four males; three of these showed immature testes, but the exact ages were not known. In the fourth case, the testes were not examined. Amongst the positive cases uncomplicated by coccidiosis, which occurred in experimentally inoculated chickens, there were but two males, one killed on the 122nd day, the other killed on the 137th day, or 132 days after inoculation. The testes in these cockerels were also immature, the seminal epithelium being almost wholly undifferentiated. By the 122nd and 137th days, spermatogenesis should be well established. Selecting from our control material healthy cockerels killed on the 109th, 111th, 122nd, 125th (2), 128th (2), 131st, 135th and 137th days, we find active spermatogenesis, with ripe spermatozoa in the tubules, in every instance. It would seem that the disease in these two cases, which was of a severe type, definitely delayed or suppressed the onset of spermatogenesis. To what extent this is a characteristic effect cannot be decided without further data.

Alimentary Tract.—No lesions have been found in proventriculus or gizzard. The great majority of the birds, both in the spontaneous and experimental cases of the disease, showed no lesions of the intestinal tract. In a few instances in which there were lymphomatous tumors of other abdominal viscera, the intestinal mucosa, especially that of the duodenum, was thickened by diffuse infiltration of mononuclear cells. Lymphomatous plaques also occurred in the subserous tissue.

The relation of worm infestation and coccidiosis to the disease will be discussed below.

Thymus, Thyroid and Parathyroid.—Examined in a few of the cases, showed no alteration.

Visceral Lymphomata.

Visceral tumors, as has been stated, were found in six of the 60 spontaneously paralysed birds. Four additional cases occurred in a group of 120 chickens used in experiments.

Histologically, these were all identical in structure. They were composed of closely packed, small round cells with deeply stained nucleus and relatively scant cytoplasm. In the more actively proliferating areas where mitotic figures were extremely numerous, the cells were larger in size, the nuclei more vesicular, with distinct chromatin structure, and the cytoplasm more abundant (Fig. 15). Such cells are perhaps comparable to the larger lymphoid elements present in the centers of the germinal follicles.

With special stains (Foot's modification of the Bielschowsky method, *J. Lab. and Clin. Med.*, 1924, ix, 3), a delicate reticulum surrounding almost every individual cell was demonstrated.

The manner of growth of these tumors into adjacent healthy tissues was characteristic of that of the malignant lymphoblastomata. The cells invaded the stroma of the organs, pushing apart the parenchymal elements, and eventually replacing them completely. Thus, in the kidney, one found glomeruli and tubules widely separated by dense masses of the tumor cells, but showing little or no degenerative change. So, too, in the ovary, liver and lung, the normal elements remained intact until entirely replaced by the tumor tissue.

Necroses occurred in some of the larger tumor masses, in which case there usually remained a broad collar of living cells about the blood vessels.

The ovary has been the apparent site of origin of the main tumor mass in all instances, no males being included in our tumor cases. The growths have shown regional invasiveness into oviduct, kidney, adrenal, adjacent fat and muscle; in one case, the tumor grew directly through the vertebral column into the spinal cord. In several of the cases, there have been nodules in the liver, lungs, peritoneum and other situations. Whether these were true metastases, or whether the agent which incited the local proliferation of lymphoid cells in the ovary produced the same effect upon the lymphoid tissue in other situations, it is impossible to say.

What does seem plain, however, is the fact that these visceral lymphomata—in spite of their apparently neoplastic appearance—are but a manifestation of the same unknown agent which brings about the infiltrations in the nervous tissues. This is very clearly shown in the case alluded to, in which the ovarian tumor had penetrated the

spinal canal. In the region where the growth had invaded the cord, the closely packed cells with numerous mitoses destroyed and replaced the nervous tissue after the manner of a malignant neoplasm. But as one passed away from this site, the cellular infiltrations became distinctly perivascular, and in the remainder of the cord and brain, the lesions were identical with those found in the paralysed birds in which no tumorous growth was associated with the nervous lesions. The scattering infiltrations of the peripheral nerves were also like those usually found in the paralysis chickens, and, as is shown by our tabulated protocols, they were present in every case of visceral lymphomatosis included in our material.

We shall reserve for our final discussion the interpretation of this interesting association.

Because of the obvious resemblance of the lesions to leucemic infiltrations, careful attention was given to the blood within the vascular channels. In no instance did there appear to be an excessive proportion of lymphoid cells or other leucocytic elements, even in situations where the infiltration of the tissues was most intense. Furthermore, a study of stained smears, from paralysed birds and healthy controls, showed no significant differences in the percentage of the various types of leucocytes. Time did not permit a systematic study of the blood, but even with the data at hand, it seemed that the lesions were not associated with a leucemic blood picture.[1] That the cells forming the infiltration are not derived wholly from the circulating blood is shown by the fact that the capillaries in the infiltrated areas contain no excessive number of leucocytes, and the occurrence of numerous mitoses is proof that the cells proliferate *in situ*.

Lesions Encountered in Non-Paralysed Chickens.

One of the puzzling facts which has made the experimental study of the disease very confusing and difficult, is the frequent occurrence of lymphoid infiltrations in the nervous tissue of chickens which are not paralysed, and which betray no clinical evidence of disease. While it is true that in most of these apparently healthy birds, the

[1] Through the kindness of Dr. H. F. Pierce, we have had the opportunity to compare our blood smears with two cases of fowl leucemia, from the material of Professor Ellermann of Copenhagen.

lesions are trifling, and indeed may fall within the range of normal variation, in others they are so intense and widespread that they cannot but be regarded as pathological. It has thus been of interest to study carefully birds from various sources, so as to obtain a clear idea of what one may consider "normal."

In 32 non-paralysed birds of various ages, and from various localities, lymphoid infiltrations of the nervous system were found in ten—approximately one-third of the cases. The brain was most frequently affected, the spinal cord next and in only one case were infiltrations present in the peripheral nerves.

Fifteen of the 32 examined came from farms or experiment stations where paralysis had occurred during the present year.[2] Lesions were

TABLE I.

Occurrence of Lesions in Non-Paralysed Chickens.

Source	Number examined	Lesions			
		Brain	Cord	Dorsal ganglia	Nerves
From localities where paralysis occurs.....	15	6	5	1	1
From localities where paralysis is absent....	17	6	5	2	—
Total.....	32	12	10	3	1

found in six of these fifteen; or if we exclude two which were less than 2 months old, in five out of seven.

Seventeen birds were obtained from sources where paralysis is definitely stated not to occur. In this group, infiltrations occurred in five birds.

From these small numbers, it is impossible to draw conclusions as to the comparative incidence of lesions in non-paralysed chickens from affected and unaffected localities. One may, however, make the definite assertion that perivascular lymphoid infiltrations of the brain and cord are often found in seemingly healthy chickens from sources where paralysis is known not to occur, and in certain instances they may be of considerable intensity and wide distribution.

There is no qualitative distinction between the infiltrations found

[2] In one instance, no data as to the source of the chicken were obtainable.

in the healthy birds and in those with frank paralysis; and it would seem that only when the infiltrations implicate the peripheral nerves to such a degree as to block motor impulses, or to bring about actual destruction of nerve fibers, do paralytic symptoms become manifest. One is driven to the conclusion that the condition exists in masked form in a very considerable number of seemingly healthy chickens. We cannot, from our experience, state whether or not these birds with unrevealed lesions are on their way to develop obvious paralysis. The fact that such cases have been found on farms where paralysis does not occur suggests that the birds with mild lesions are immune to the more severe forms of the disease.

The question may be raised at this point as to whether the presence of lymphoid tissue about the meningeal and cerebral vessels may not, after all, be a normal histological feature in the chicken. It is not easy to reach a definite decision as to this, and we have found no reference in the literature to guide us. In a certain number of birds, a thorough and systematic study of numerous sections from all parts of the brain and cord has failed to disclose any perivascular lymphoid tissue. On the other hand, as has been indicated, we have frequently found small lymphoid nodules in the walls of the cerebral and spinal vessels, and in the dorsal root ganglia, in well nourished, healthy stock from unaffected sources. Such small compact focal masses of lymphoid cells have been arbitrarily regarded within the range of "normality." The line between these and the milder grades of pathological infiltration, it must be admitted, is not a sharp one, and the difficulty becomes especially troublesome in attempting to interpret the effects of experimental inoculation.

Bacteriology. Attempts to Demonstrate an Infective Microorganism.

Efforts to identify an infecting organism in smears and sections of the brain, spinal cord, peripheral nerves and heart blood have thus far proved unavailing. Sections have been stained by the Gram-Weigert method, with toluidine blue, Giemsa's method, Wright's stain, Levaditi's method and Jahnel's modification of Levaditi's method for spirochetes. None of these procedures has disclosed any structures which can with reasonable certainty be recognized as bacterial or protozoan. We have had in mind the possibility of

rickettsial infection, and we have carefully searched our preparations for intranuclear or cytoplasmic inclusions.

We have made numerous attempts to obtain cultures of organisms from the nerve tissue of paralysed chickens. Among the media used were peptone and meat extract broth, with and without addition of glucose; plain and glucose agar, with and without addition of inactivated chicken serum or whole blood, and adjusted to various pH ranging from 7.4 to 6.0; beer-wort agar; chopped chicken meat broth, and potato. Cultures were incubated at 37° and at room temperature. Following a suggestion of Dr. Rettger, we tested the possible stimulating effect of an atmosphere of approximately 10 per cent CO_2. Although diphtheroids, sarcinæ, yeasts and torulæ were occasionally obtained, none of the strains isolated appeared to be of etiological significance and they were regarded as accidental contaminants.

TABLE II.

	Scrapings + Sections +	Scrapings − Sections −	Scrapings + Sections −	Scrapings − Sections +
Duodenum	10	48	2	1
Cecum	12	7	3	0

Fowl Paralysis and Coccidiosis.

The view that coccidial infection may play a rôle in the etiology of paralysis has been expressed by a number of poultrymen and experiment station workers. Thus Beach and Davis (7) describe and picture (Fig. 3 of their article) paralysis as a form of chronic coccidiosis occurring in older birds between the ages of 4 and 8 months. The authors do not refer to "fowl paralysis" as an independent disease, but appear to regard it merely as a late symptom of the coccidial infection. The opposite point of view is represented by May, Tittsler and Goodner (5), who state that coccidia are frequently present in paralysed birds but not in all cases; and that birds may die from coccidiosis without exhibiting symptoms of paralysis.

Our material, we believe, affords a decisive answer. As a routine, sections were taken from duodenum and ceca in practically every case examined; during the past summer, we have also checked the

histological findings by the examination of fresh scrapings of the duodenal and cecal mucosæ. There is, in general, close agreement between the findings of coccidia in fresh preparations and in the stained tissue sections. This is brought out in Table II.

Thus, in only six out of 83 examinations was there any discrepancy as regards the presence or absence of coccidia between fresh preparations and the stained sections. From this one may conclude that the absence of coccidia in histological sections is strong presumptive evidence against the existence of coccidial infection.

In 60 spontaneous cases of paralysis, coccidia were found in the sections in ten, or roughly, 16 per cent; in 33 non-paralysed birds from various sources, coccidia occurred in four, or approximately 12 per cent. Obviously, there is no significant difference in the two groups.

Our experimental material is also instructive. In Experiment VI, only one of thirteen cases of paralysis was infected with coccidia. In 83 chickens which did not develop paralysis, coccidial infection occurred in eleven, or roughly, 13 per cent, about the same ratio as in the non-experimental material. In Experiment VII, coccidiosis appeared in both inoculated and control animals during the 3rd and 4th months. Of the four positive cases, two showed coccidia, and two were free. Fifteen of the non-paralysed chickens were affected, and 33 were free. The higher incidence of coccidiosis in this experiment may be attributed to the crowding and close confinement.

From the foregoing data, it is very evident that there is no correlation whatever between infection with *Eimeria avium* and the incidence of fowl paralysis.

DISCUSSION.

The disease as thus described is one with striking and characteristic pathology, and one which has no close counterpart, so far as we are aware, in mammalian pathology. The essential changes are cellular infiltration of the nervous tissues, peripheral nerve trunks of the extremities and viscera, spinal ganglia and cord—less strikingly of the brain and rarely of the cranial nerves. In the nerves, the cells, which are predominantly of the lymphoid type, but mixed with plasma cells and clasmatocytes, push apart the nerve fibers and

eventually cause considerable destruction of the myelin. The axis cylinders tend to be preserved so that it is not surprising that paralysis is rarely complete. That sensation as well as motion is affected, is evident from the involvement of the spinal ganglia and posterior spinal roots in most of the cases.

In the cord and brain the infiltrations are largely confined to the vicinity of the vessels and the lesions often bear a close resemblance to those found in various types of mammalian encephalomyelitis. Although these lesions of the nervous system are the most distinctive features of the disease, and probably the ones responsible for the more obvious clinical symptoms, it seems probable that the massive lymphomatous infiltrations and growths occurring in the ovary and other viscera are integral features of the disease. They have been present—and we refer here only to the grossly evident, tumor-like growths—in 10 per cent of our spontaneous cases; and they have developed, as will be pointed out in a following paper, in fowl experimentally inoculated with suspensions of brain and cord from paralysed fowl. Although these masses often take on the character of a malignant neoplastic growth, pushing aside and invading normal structures, their cytological composition, and the frequent continuity of the neoplastic tissue with the characteristic accumulation in the nervous tissues make it highly probable that the same agent or stimulus is concerned.

This brings up the difficult questions of the relation of this disease to the malignant lymphoblastomata on the one hand, and to the leucemias and pseudoleucemias on the other.

Numerous tumors in fowls variously termed lymphoma, lymphosarcoma and small round cell sarcoma have been described.

In certain cases these have been associated with a leucemic blood picture, and considerable evidence has been presented by Ellermann and Bangs (8), Ellermann (9), Hirschfeld and Jacoby (10), Schmeisser (11) and others that the leucemia of fowls is a transmissible disease. Of the non-leucemic lymphomatous cases, we may cite those reported by Hathaway (12), Butterfield (13), Tyzzer and Ordway (14), Wernicke (15), Joest and Ernesti (16), Pentimalli (17), Beatti (18), Hobmeyer (19), Elsner (20), Schuchmann (21) and Michalka (22). It is unnecessary to review this literature in detail. That the disease which we have studied is not a form of leucemia has been shown by the examination of blood, smears and by the absence of enlargement of the spleen and liver, which has been a characteristic feature of the reported cases.

It has seemed to us that the diagnosis of "pseudoleucemia" and "leucanemia" used by Ellermann to describe cases in which lymphoid or myeloid infiltrations were present in the viscera without a leucemic blood picture, must be accepted with considerable reserve. Ellermann regards such cases as being produced by the injection of his leucemic virus, and includes them amongst the positives. In our experience, such visceral infiltrations occur in practically every chicken, although there is considerable individual variation in the amount of lymphoid or myeloid tissue present in the different organs. Neither Ellermann nor subsequent workers with fowl leucemia appear to have made a systematic study of control "normal" material. It is regrettable also, that none of the investigators of fowl leucemia has included a study of the nervous system. We cannot state, therefore, whether brain, cord and nerves in this disease may show lymphoid infiltrations such as we have described in fowl paralysis. That such infiltrations may be found in human leucemias, is well known; 30 such cases have recently been collected from the literature by Fried (23). That they cannot involve the peripheral nerves to the degree found in the paralysis of chickens, is obvious from the absence of neuritic symptoms in leucemic birds.

Whether some of the cases which have been described as "pseudoleucemia" may have been instances of paralysis with visceral infiltrations, it is impossible to say. The literature contains no references to a careful study of the nervous system in these cases. Only in the case reported by Beatti from Buenos Aires, is it recorded that the chicken during life could not stand erect, but rested on the right foot, using the left wing as support. The cause for this was a lymphoblastomatous mass in the lumbosacral region, infiltrating the kidney, and invading the lumbosacral plexus. Tumor cells were distributed among the nerve fibers. There was no leucemic blood picture. It is probable that this was a true case of fowl paralysis, of the type associated with regional lymphomatous growth, and that other cases in the literature, if the nervous system had been carefully studied, would have revealed themselves as examples of this disease.

A striking association between paralysis and a high tumor incidence (12.9 per cent) was in evidence also in the "farm flock" cited by Schneider (1926) (24). From a study of material supplied to us, we have learned that paralysis was prevalent in this particular flock.

In the 60 cases of spontaneous paralysis here reported, macroscopic ovarian tumors, usually with lymphomata also in the other viscera, were found in six instances or 10 per cent and we have found a similar high incidence in our experimental chickens. The abnormally high

rate in our paralysis material is in itself suggestive of a correlation, and we have given pathological evidence for the view that the lymphomatous masses in the ovary and other viscera are no different in their cytological composition from the infiltrations found in the nervous tissues. Our material does not permit us to state whether all cases of visceral lymphomatosis in fowls are accompanied by lesions of the nervous system, and this question demands further investigation.

Our studies at least indicate that the agent which is responsible for the pathological changes in the nervous system is not exclusively neurotropic; it may in certain cases stimulate the proliferation of lymphoid cells in the viscera, and to a degree which makes them take on the morphological character of a neoplasm. One of the most interesting and as yet, wholly obscure phases of our problem, is the undoubted transition between the lesions which histologically appear to be inflammatory and those which assume the character of a true neoplasm.

Nomenclature.

No satisfactory designation for this disease has yet been suggested. The term "polyneuritis" used by Marek in 1907, is open to several objections—it is not sufficiently inclusive, leaving out of account the lesions of the cord, ganglia and brain; and it does not distinguish this disease from the nutritional polyneuritis gallinarum, which is etiologically and pathologically unrelated. Kaupp, and May, Tittsler and Goodner speak simply of "paralysis of the domestic fowl"—a term which is unsatisfactory, since paralysis is but a symptom, and one which is inconstantly present in this disease, and which may be due to a variety of other disorders. The term "neuritis" is used by Doyle, and is open to the same objections as the term "polyneuritis." Van der Walle and Winkler-Junius suggest the name "neuromyelitis gallinarum" to differentiate this condition from the nutritional polyneuritis.

It is perhaps not possible to find an altogether satisfactory name for this disease until the cause of it shall have been definitely established. The outstanding pathological features, as we have seen, are the lymphoid infiltrations of the peripheral nerves, and the frequently associated lymphomatous growths in other situations. We wish, therefore, to propose tentatively the term neurolymphomatosis gallinarum, as indicating more specifically than any of the

terms previously suggested, the most striking pathological features of the disease.

CONCLUSIONS.

1. Fowl paralysis (neurolymphomatosis gallinarum) is a disease entity, with characteristic clinical and pathological features.

2. The disease occurs in all parts of the United States, Holland, Austria and probably South America.

3. The disease appears to be endemic in certain foci. Having once appeared, the disease tends to persist through successive years.

4. It occurs with about equal frequency in both sexes; all common breeds may be affected.

5. Symptoms appear between the 3rd and 18th months. Typical clinical cases have not been observed outside of these limits.

6. The conspicuous symptoms are (*a*) asymmetrical, partial and progressive paralysis of the wings and both legs, and rarely of neck muscles; (*b*) occasional grey discoloration of iris, with blindness. Nutrition is usually preserved.

7. The duration is variable; the outcome is usually fatal, but spontaneous recovery may rarely occur.

8. The principal pathological changes are found in the nervous system. In the peripheral nerves, the essential feature is an intense infiltration of lymphoid, plasma cells, and large mononuclears. This is accompanied by a myelin degeneration in the more advanced lesions, but the cellular infiltrations appear to precede the degenerative changes. In brain, cord and meninges, there are similar infiltrations predominantly perivascular.

Infiltrations of the iris with lymphoid and plasma cells are found in the cases showing gross discoloration of the iris.

Visceral lymphomata, originating usually in the ovary, are associated in a certain percentage of the cases. Evidence is presented in favor of the view that this association is not accidental, and that the lymphomata are a manifestation of the disease.

9. Infiltrations of the spinal cord and brain, rarely of the peripheral nerves, are frequently present in birds showing no clinical symptoms. These are interpreted as mild cases of the same disease.

10. No microorganisms of etiological significance have been demonstrated in the tissues or by cultural methods.

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EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. Fowl paralysis. Showing weakness of legs and drooping of left wing.

FIG. 2. Fowl paralysis. Terminal stage, complete prostration.

FIG. 3. Fowl paralysis. Dissection of spinal cord and brachial plexus. On the right side there is massive nodular thickening of the nerve trunks, extending into the corresponding segment of the cord.

PLATE 7.

FIG. 4. Section through sciatic nerve. Intense mononuclear infiltration of upper bundle, moderate infiltration of lower.

FIG. 5. Fowl paralysis. Infiltration of peripheral nerve. High power.

PLATE 8.

FIG. 6. Fowl paralysis. Section of sciatic nerve, showing intense cellular infiltration. Note nodular foci.

FIG. 7. (*A*) Brachial plexus. Normal. (*B*) Advanced paralysis. Myelin degeneration, fibrosis, mononuclear infiltration.

PLATE 9.

FIG. 8. Typical paralysis. Sciatic nerve. Pal-Weigert stain, showing advanced degeneration of myelin sheaths.

FIG. 9. Typical paralysis. Sciatic nerve stained by the Rio del Hortega silver carbonate method. Persistence of neuraxones in infiltrated nerve.

PLATE 10.

FIG. 10. Low power photograph of spinal cord with attached ganglia and nerve roots, showing asymmetrical enlargement and extension of infiltration into adjacent tissue of cord.

FIG. 11. Spinal ganglion, showing lymphoid infiltration between ganglion cells.

PLATE 11.

FIG. 12. Fowl paralysis. Infiltration of sympathetic ganglia and nerves about adrenal.

FIG. 13. Section through cornea, anterior chamber of eye, iris and lens. Note cellular infiltration of iris.

PLATE 12.

FIG. 14. Fowl paralysis. Massive lymphoid infiltrations in liver.

FIG. 15. Fowl paralysis. Section of ovarian tumor, associated with typical lesions of the nervous system. Note numerous mitoses. High power.

FIG. 1.

FIG. 2.

FIG. 3.

(Pappenheimer *et al.*: Fowl paralysis. I.)

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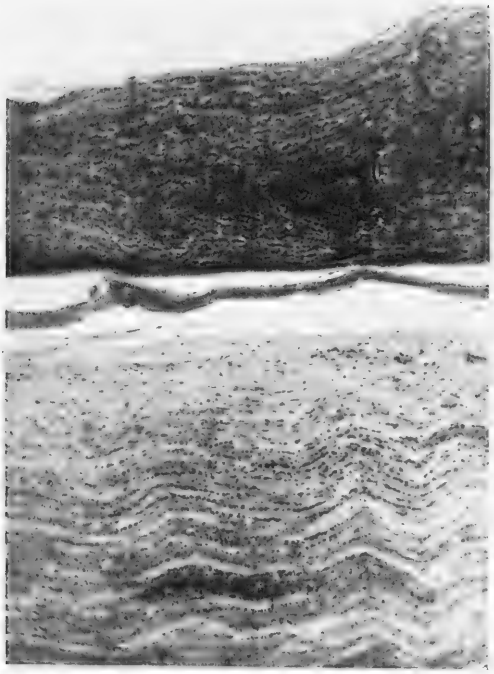

FIG. 4.

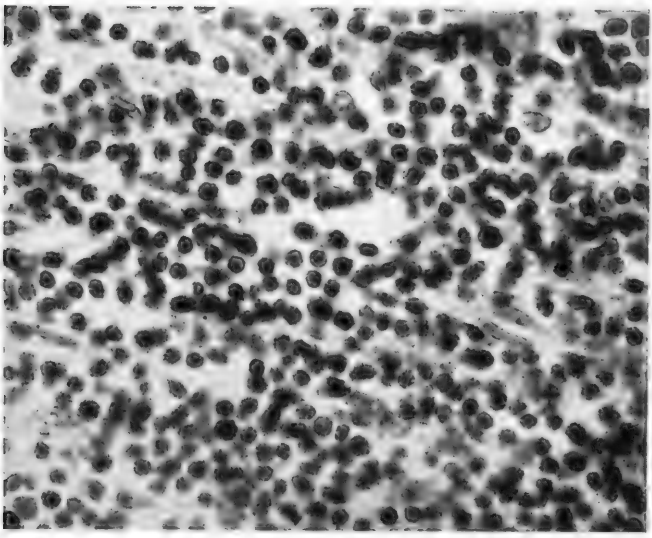

FIG. 5.

(Pappenheimer *et al.*: Fowl paralysis. I.)

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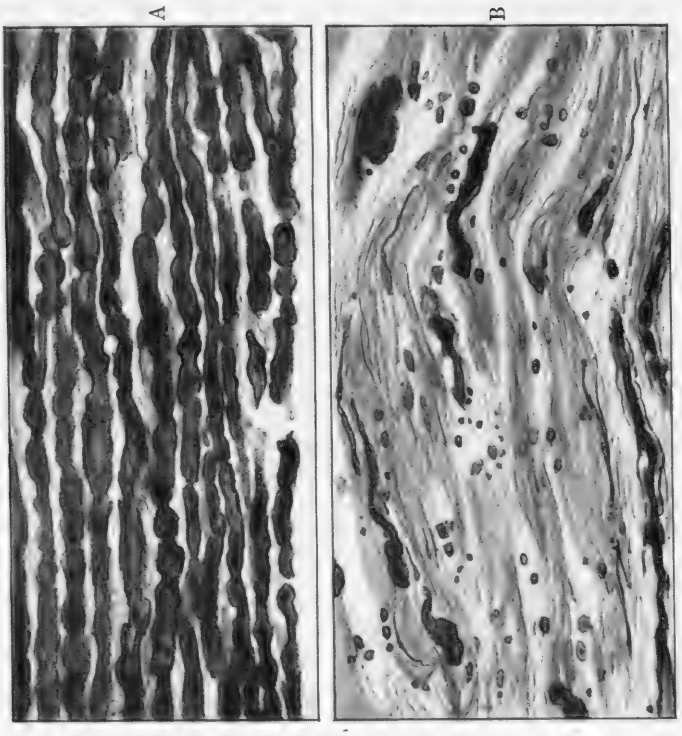

FIG. 7.

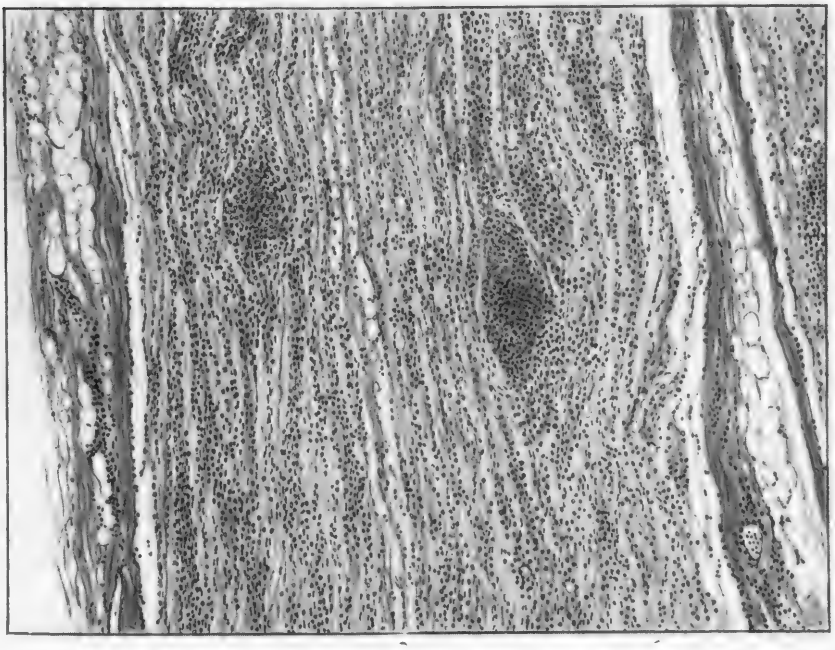

FIG. 6.

(Pappenheimer *et al.*: Fowl paralysis. I.)

FIG. 8.

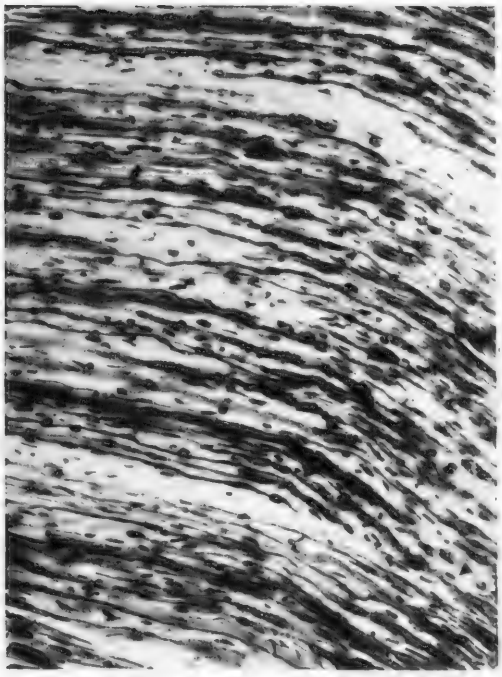

FIG. 9.

(Pappenheimer *et al.*: Fowl paralysis. I.)

FIG. 10.

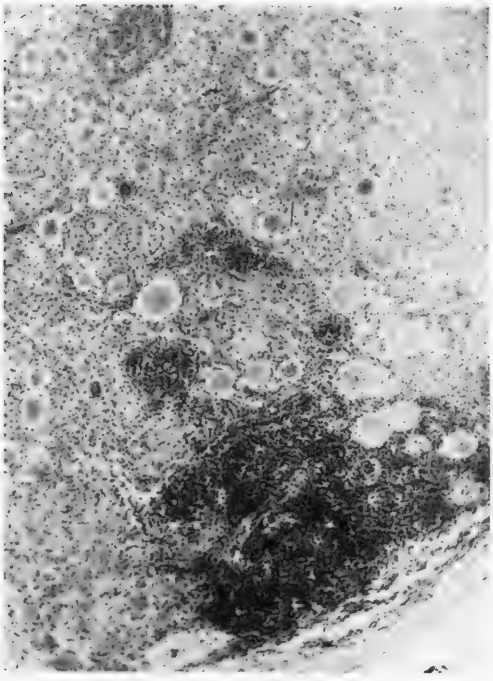

FIG. 11.

(Pappenheimer *et al.*: Fowl paralysis. I.)

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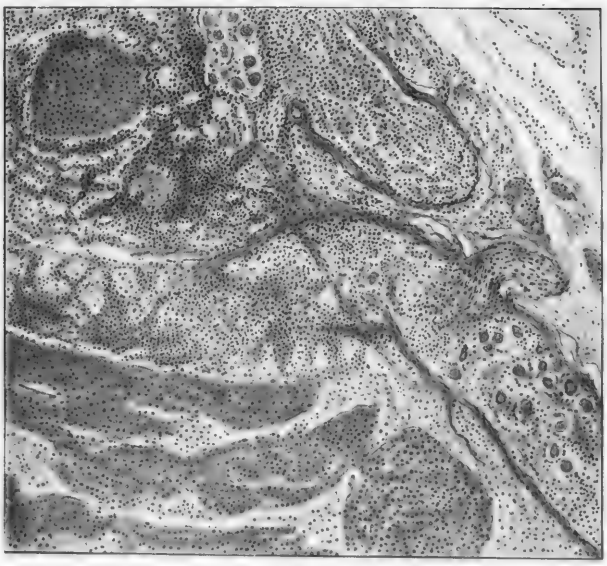

FIG. 12.

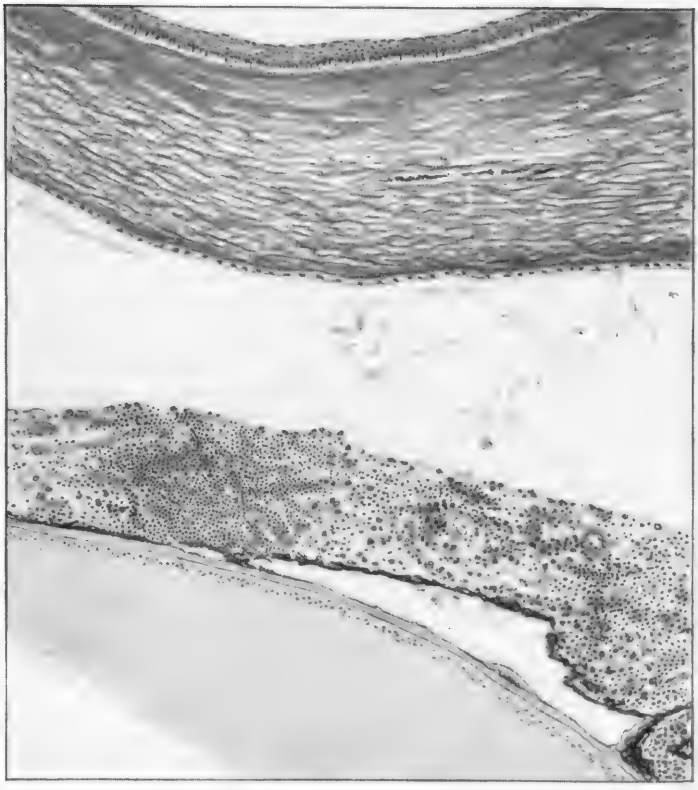

FIG. 13.

(Pappenheimer *et al.*: Fowl paralysis. I.)

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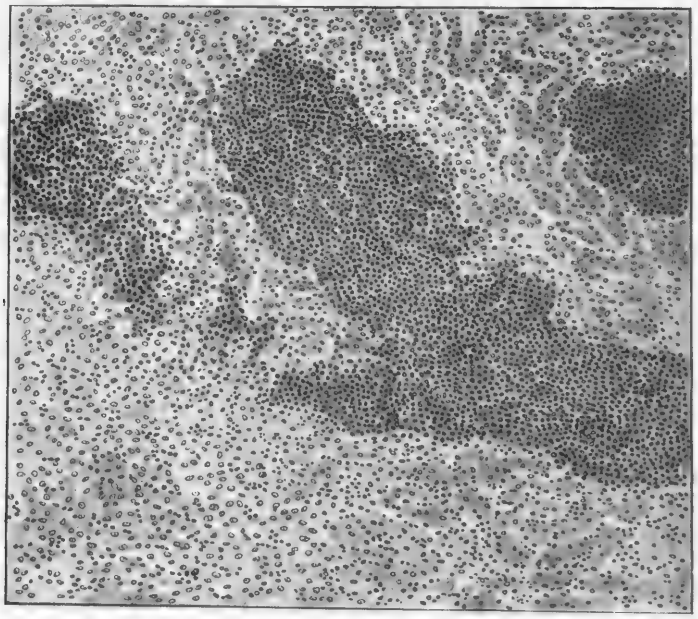

FIG. 14.

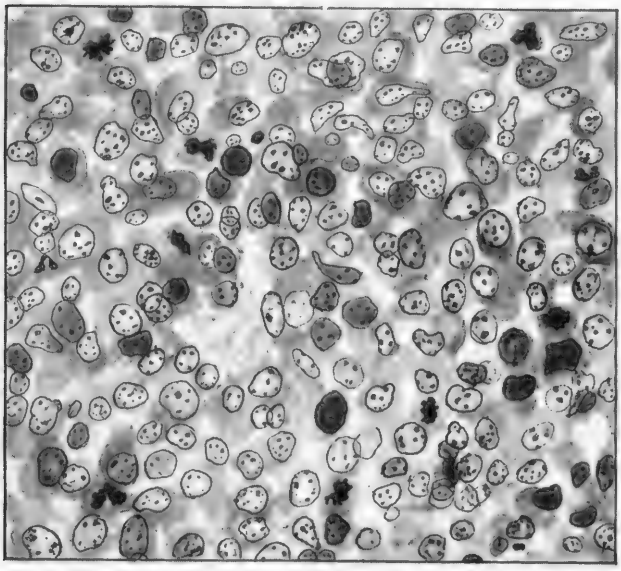

FIG. 15.

(Pappenheimer *et al.*: Fowl paralysis. I.)

STUDIES ON FOWL PARALYSIS (NEUROLYMPHOMATOSIS GALLINARUM).*

II. Transmission Experiments.

By ALWIN M. PAPPENHEIMER, M.D., LESLIE C. DUNN, Ph.D., and S. M. SEIDLIN, M.D.

(From the Storrs Agricultural Experiment Station, Storrs, and the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York.)

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In a foregoing article, a brief description was given of the symptoms and pathology of the disease known as fowl paralysis. Although efforts to demonstrate a causative organism in the tissues or by cultural methods were fruitless, it seemed fundamentally important to determine whether or not the disease was transmissible from one chicken to another under experimental conditions. The present article presents, in condensed form, experiments which have been carried out toward this end.

Previous workers have made efforts to establish the transmissibility of this disease. Kaupp (1), May, Tittsler and Goodner (2) and Doyle (3) all report negative experiments—using a variety of methods and material for inoculation. Van der Walle and Winkler-Junius (4), however, report the following experiment which is regarded by them as establishing the infectious nature of the disease. A hen fed with maize and barley and given water out of the well of the farm where paralysis occurred, showed symptoms of paralysis after 2 months. Another hen, fed with muscles from an affected hen, became atactic after 5 months. The blood of this hen, kept anaerobically for 18 days at 37° was injected into a healthy bird. A culture of bouillon was made from the blood of the latter, filtered through a Chamberland filter and injected intravenously into another hen. This showed

* This work was aided by a generous grant from the Mount Hope Farm, Williamstown, Mass. The writers wish also to express their grateful acknowledgment to the Scientific Director, Dr. D. H. Goodale, for material assistance and valuable suggestions. We have also received material and interested cooperation from numerous other sources. Although it is impossible here to make individual acknowledgments, the writers wish to express their thanks for this assistance.

symptoms of paralysis after 2 months, but recovered after 2 weeks. Another hen injected intraperitoneally with blood of a diseased bird, developed paralysis after 46 days. Five birds kept in the same pen with the affected birds, failed to develop the disease. The authors concluded from the foregoing experiment—in our opinion from insufficient evidence—that the disease is an infection due to a filtrable virus.

Our preliminary experiments were begun in June, 1925. At that time, we did not realize how frequently lesions of the central nervous system may be found in apparently normal chickens, nor were we aware of the long period which may elapse between the inoculation and the appearance of symptoms. In spite of these difficulties, a careful histological examination of the first twenty inoculated birds (Experiments I, II, III, IV) showed a suggestively higher incidence of lesions in brain, cord, spinal ganglia and more particularly in the peripheral nerves than was found in a series of uninoculated, non-paralysed controls. Thus, infiltrations of the sciatics or brachial plexus were present in 40 per cent of the 20 experimental animals, and in but one (3 per cent) of the 32 controls.

In subsequent experiments, inoculations were made by preference into newly hatched chicks, in the hope that spontaneous infection during the long developmental period of the disease might be avoided, and that the chicks might be less refractory at an early age.

Experiment V.—August 7, 1925. Eight 10 day old chicks were inoculated *subdurally* with unfiltered brain suspension of a typical advanced paralysis case. Eight chicks of the same hatching were kept as controls under identical experimental conditions. They were raised indoors on pine shavings; cod liver oil was included in the diet to prevent leg weakness.

One of the inoculated chickens was found dead and discarded without examination. The remaining seven were killed, 10, 14, 24, 34, 53, 62 and 120 days after inoculation, a control being sacrificed at the same time. One chick was also killed for examination at the beginning of the experiment.

The results of our histological studies in this series are summarized in Table I.

Lesions of the nervous system were found in all but one of the inoculated birds, and in but one of the controls. Only one inoculated chick was permitted to survive to an age at which clinical paralysis may be expected to appear. This chicken developed weakness of the legs 2 weeks before death, which did not improve with cod liver oil;

it was killed on the 120th day. There were found infiltrations of the brain, cord, ganglia and peripheral nerves, slight in intensity, but of the usual character, and this may be regarded as a positive case.

Experiment VI.—In this experiment, the eggs were obtained from a farm in New Hampshire where in a flock of 6000, no clinical case of paralysis had been observed. The eggs were hatched in the laboratory of the Storrs Experiment Station.

One hundred and twenty 5 day old chicks were divided into four lots of 30 each.

October 27, 1925. Lot 1 was injected with 0.1 cc. of unfiltered brain suspension, in 0.85 per cent NaCl, of a typical paralysed chicken.

Lot 2 received 0.1 cc. of brain suspension of a normal 5 day old chick.

Lot 3 received 0.1 cc. of a 24 hour old anaerobic "culture" of brain and cord of typical paralysed fowl (F-301), with Hartley broth used according to the tech-

TABLE I.

10 day old chicks inoculated subdurally with saline suspension of brain of paralysed chicken. Occurrence of lesions compared with controls from same hatch. Killed at intervals from 10 to 122 days.

	Lesions			
	Brain	Cord	Ganglia	Nerves
Inoculated.....7	6	4	2	1
Controls.....8	1	1	2	0

nique of Gye for the demonstration of a filtrable agent in avian and mammalian tumors. Anaerobiosis in the hydrogen jar was not complete, the test solution of methylene blue failing to be completely reduced.

Lot 4 was kept as controls, and was not inoculated.

The chicks were raised indoors on a table covered with pine shavings and sand. The different lots were separated by wire netting partitions. Drinking cups and feed troughs were kept separate, and disinfected daily. Cod liver oil was incorporated in the ration to prevent leg weakness. Weekly weighings were continued until the loss of a large number of chicks through an epidemic of bronchitis during the latter part of December and early January made the weight data of the survivors statistically valueless.

It may be stated here that the mean weight of each of the four groups during the first 9 weeks, before the onset of the epidemic, did not differ significantly, and was approximately normal for the conditions and breed. In other words, the inoculation *per se* had no effect upon the general development and nutrition of the birds.

The chickens that died spontaneously or that were killed for examination were subjected to a careful gross and histologic study. Blocks were taken from all parts of the brain; the entire spinal cord was removed, divided into segments and longitudinal sections of each block studied, together with some of the attached spinal ganglia. Sections also were taken from each brachial plexus, from the sciatic nerves at various levels and from lung, liver, spleen, myocardium, kidneys, pancreas, duodenum, ceca, adrenals and gonads.

100 birds from this experiment were examined after appropriate intervals, distributed as follows: Lot 1, 28; Lot 2, 24; Lot 3, 21; Lot 4, 27.

The results may be briefly summarized as follows:

Lot 1.—Seven chickens of this group (25 per cent) inoculated with suspension of paralysis brain and cord developed characteristic lesions of the disease, being sacrificed or dying spontaneously after 64, 71, 73, 75, 132, 190 and 276 days after injection.

Lot 2.—Inoculated with brains of healthy 5 day chicks. Of the 24 birds examined, two presented, after 156 and 176 days, the typical anatomical picture of paralysis, in the case of one chicken associated with visceral lymphomatosis. A third showed atypical lesions, not involving the peripheral nerves.

Lot 3.—Inoculated with 24 hour anaerobic Hartley broth "culture." Out of 21 birds examined, only one, killed after 150 days, developed symptoms and lesions of paralysis.

Lot 4.—Uninoculated controls. Two definitely positive cases occurred in this group, one on the 65th day, the other on the 86th day after the beginning of the experiment. A third apparently healthy cock, killed 175 days after inoculation, showed minimal infiltration of the peripheral nerves and was regarded as a doubtful case.

There is thus a definitely higher incidence of the disease in Lot 1, inoculated with material from the paralysed bird, than in the three other control groups. Expressed percentally upon the basis of the pathological findings, there are 25 per cent of positives in Lot 1, as against 8.3, 4.8 and 7.5 per cent in Lots 2, 3 and 4. This difference suggests, but does not convincingly establish the transmissibility of the disease.

Under ideal conditions, each individual bird should have been strictly segregated throughout the long experimental period, so that any possibility of secondary infection, either from other inoculated

birds of the same group, or from extraneous sources, would be so far as possible excluded. It was not practicable to carry out a large scale experiment under these rigid conditions. While the effort was made to segregate the different groups, it must be admitted that the possibility of infection from bird to bird or through intermediary sources was by no means adequately controlled.

Under these circumstances, the occurrence of a certain number of cases in the control groups is not surprising. In spite of the technical limitations in the conduct of the experiment, the considerably higher incidence of the disease in Group 1, in which the number of positive cases surpassed that in the other groups combined, must be taken to mean that the inoculation of material from paralysed birds favored the subsequent development of the disease.

It is clear, however, that only a certain proportion of newly hatched chicks are susceptible; in this experiment, only one in four later manifested the disease in its typical form.

Assuming the existence of a specific virus, failure to acquire the disease may theoretically be explained in one of several ways. There may have been an uneven distribution of the infective material in the inoculum. Resistance to the graver form of the disease may have been acquired through a mild spontaneous infection picked up during the early stages of the experiment. There may be inherited factors influencing resistance or susceptibility.

One cannot profitably discuss these possibilities with the facts at hand. But another question arises which bears even more closely upon the interpretation of the experiment. What is the significance of the slight infiltrations of the brain and cord, found in so large a proportion of both inoculated and control chickens? Do they signify the existence of the disease in a mild form, undisclosed by clinical symptoms? If so, are we justified in regarding as positive only those cases which develop pronounced and intense lesions of the peripheral nerves?

Excluding the frankly typical positive cases in each group, we find the infiltrations of the brain and cord distributed amongst the four groups as shown in Table II.

The slightly higher incidence of brain lesions in Group 1 is counterbalanced by the lower incidence of cord lesions. With the small

numbers involved, and the possibility of overlooking isolated lesions in routine examination, it seems likely that the differences are accidental, and that the lesions are fairly uniformly distributed throughout the four groups.

Table III shows the distribution when not only birds with pronounced lesions involving the peripheral nerves, but also those showing slight infiltrations limited to the brain and cord, are included amongst the positive cases.

Although there is again a higher incidence of lesions in Group 1, the

TABLE II.

	Brain + or ±	−	Cord + or ±	−	Brain Per cent +	Cord Per cent +
Lot 1	13	6	5	14	68	26
Lot 2	10	11	8	13	47	38
Lot 3	10	9	8	10	52	44
Lot 4	11	14	9	16	44	36

TABLE III.

	Positive	Negative	Percentage positive
Group 1	21	6	78
Group 2	16	8	66
Group 3	14	7	66
Group 4	15	12	55

difference does not appear to be very striking, nor perhaps beyond the range of chance distribution; so that if we regard the chickens with mild lesions as being infected with a mild form of the disease, it would be impossible to ascribe any effect to the inoculation other than a slightly increased susceptibility to the graver form of the disease. In other words, from 55 to 65 per cent of the chickens acquired the disease naturally during the experimental period; and an additional 10 per cent acquired it as a result of inoculation. This is indeed a puzzling situation.

It may be suggested that the slight lesions found in so large a proportion of the birds represent a reaction of the tissues to a different

agent from that causing the more intense lesions found in typical paralysis cases. This possibility cannot be controverted so long as we are forced to depend upon the histological lesions for a recognition of the disease. But our material includes an unbroken series of transitions between the slightest lesions and those which approach true neoplasms in the intensity of the cell proliferations, so that it seems logical, for the present, to assume that we are dealing with the same inciting cause acting upon organisms of varying reactivity.

Experiment VII.—In the hope of eliminating so far as practicable, the possibility of extraneous infection during the long experimental period, another experiment was carried out under the following, more rigid, conditions.

Fertile eggs were obtained from the same source as those used in the preceding experiment. They were hatched at Storrs in a Blue Hen incubator in a separate disinfected compartment. The date of hatching was April 28, 1926. They were kept in the incubator until April 30, on which day 18 were inoculated subdurally and 15 intramuscularly into the left leg with material from a typically paralysed bird. The cord, enlarged cervical ganglia and left sciatic nerves were asceptically removed, ground in 10 cc. of 0.85 per cent salt solution, centrifuged and the turbid supernatant fluid used for injection. Of this fluid, 0.1 cc. was injected subdurally, and 0.2 cc. intramuscularly.

After injection, the chicks were replaced in the incubator, and on the following day removed to a farm situated at a distance of a mile from the Experiment Station. At this time there were no chickens on this farm, nor within a radius of about a quarter of a mile. The experimental chicks were placed in charge of Mr. Milton Moore, with the arrangement that he was scrupulously to avoid contact with other chickens throughout the course of the experiment.

The brooders were not ready until May 3, so that for 3 days the chicks were kept in the house in a separate room from the controls. On that day, they were transferred to a specially constructed outdoor brooder house, with electric hovers. These were so designed that food and water could be given and bedding changed without handling the birds. A sun porch screened with fine mesh wire was attached to the end of the brooder house, and covered with burlap to avoid possible contamination by the droppings of wild birds. Freshly distilled water was used throughout the entire course of the experiment. The food was that used in the previous experiment, and peat moss was used for bedding. For a brief period during the 1st month, fresh grass was added as a supplement to the diet. This unfortunately introduced an unknown factor and a theoretically possible source of outside infection, but was considered necessary, because several chicks had died with extensive urate deposits in the kidneys. After the addition of grass to the food, no further trouble from this condition was experienced.

34 chicks of the same hatching were used as controls, and were kept under identical experimental conditions in a brooder house about 30 feet away.

It was deemed inadvisable to subject the chicks to the handling involved in weekly weighings. On August 23, a sample of 15 chicks of the inoculated group, and 17 of the controls were weighed. The average weight of the inoculated was 1060 gm.; of the controls, 1147 gm. This difference is probably without statistical significance.

The experiment was thus planned to exclude infection by direct contact, by contact with possible human carriers, by insects of a size larger than the fine meshed wire gauze used and by drinking water.

Results.—Of the 31 inoculated birds, three developed the disease in a pronounced and typical form, one dying spontaneously on the 78th day, and two being killed on the 68th and 117th days after having exhibited unmistakable symptoms of paralysis. At least four other chickens of the inoculated group presented lesions of sufficient gravity to warrant their inclusion amongst the positive cases. The total incidence—seven out of 31, or 22 per cent—agrees well with the incidence of positive cases in Group 1 of the previous experiment.

The control group, unfortunately, did not remain wholly free of the disease, although there were no outstanding cases comparable in severity to the three above mentioned, and but two cases which could reasonably be regarded as comparable to the four mildly positive cases amongst the inoculated birds. The incidence in the control group may thus be reckoned as two out of 32, or 6.2 per cent.

Table IV brings out the comparative incidence and severity of the nervous lesions in the two groups.

There can be no question that in this experiment there is a significantly higher incidence of infiltrations in all parts of the nervous system, and particularly in the peripheral nerves in the inoculated group. One cannot escape the conclusion that the inoculation of the supposedly infective material is in some way correlated with this higher incidence, inasmuch as the inoculation was the only known variable in the experiment.

Several points require comment. The long period which elapsed between the inoculation and the first appearance of symptoms or the development of manifest lesions is striking. In one chicken paralysis was first noted 68 days after inoculation; another chicken died on the 78th day, and the third positive case first presented symptoms on the 115th day, and was killed 2 days later. We have seen in discussing the age incidence of the spontaneous cases, that there is a refractory period of approximately 3 months after hatching, during which, in our experience, the disease does not become manifest. This freedom of young growing chicks from the clinical evidence of the disease is apparent also in our experimental cases.

Another point of interest is that in at least one of the three positive cases, the disease developed after intramuscular injection. In two of the mildly positive cases, also, the inoculation was by the intramuscular route. We may conclude, therefore, that it is not essential to introduce the supposedly infective material directly into the nervous tissue in order to produce the characteristic effect.

Experiment VIII.—The purpose of this experiment was to determine whether the disease could be transmitted by the inoculation of a cell-free Berkefeld N filtrate alone, or by a filtrate "activated" by the addition of embryonic extract.

April 5, 1927. Brain and cervical cord with attached ganglia of a paralysed White Leghorn hen (Storrs 12–014) were removed aseptically, ground in sterile Tyrode's solution (pH 7.8), centrifuged at low speed and the supernatant filtered through a tested Berkefeld N filter. Inoculations were made into 6 day Spangled

TABLE IV.

	Brain			Cords			Nerves		
	Marked	Moderate or slight	Negative	Marked	Moderate or slight	Negative	Marked	Moderate or slight	Negative
Inoculated	0	24	7	2	23	6	3	14	14
Controls	0	8	24	1	15	16	0	5	27

Hamburg chicks obtained from a commercial hatchery in Ohio. This strain was selected because it had exhibited an unusually high mortality from the spontaneous disease during the previous season. 15 chicks were inoculated subdurally with 0.1 cc. of the clear filtrate; 16 chicks received 0.1 cc. of filtrate diluted with an equal volume of freshly prepared chick embryo extract; 18 chicks were injected with 0.1 cc. of embryo extract alone, to control the possible presence of the "virus" in the embryos used to prepare the extract. The two groups receiving the injection of material from the paralysed birds were reared in a separate room at the College of Physicians and Surgeons; the control group was strictly segregated in another room of the animal house.

Two of the chickens died spontaneously after 70 and 97 days respectively, both showing a severe coccidial infection, but no symptoms or lesions of paralysis.

The remaining members of the three groups were sacrificed during the period from 115 to 124 days after inoculation. No clinical symptoms of paralysis were observed, and a very careful gross and microscopic study failed to disclose typical lesions of paralysis.

This experiment therefore offered no support for the view that the disease is carried by a "filtrable" virus. We attach only limited importance to this negative experiment, however. Apart from the complex factors which affect the filtrability of ultramicroscopic viruses, the importance of this particular experiment is limited by the choice of an alien strain of chicks for inoculation. Possibly also the dosage of "virus" was insufficient.

The addition of embryonic extract to the inoculum failed to exert an activating influence.

Experimental IX.—The material for inoculation in this experiment was obtained from a chicken hatched in the laboratory at the College of Physicians and Surgeons; injected on the 2nd day into muscle of right leg with suspension of nerve tissues and arsenious acid (1–150,000), from a typical spontaneous case of paralysis. The bird had been isolated with four other chicks of the same hatching. On the 82nd day, it was found to have definite paralysis of the right leg, with drooping of the wings. 4 days later, it was etherized and bled from the heart. Brain, cord and peripheral nerves were removed asceptically and mashed in 50 per cent glycerol. The material was kept in the ice box for 9 days.

Thirteen 4 day old chicks were inoculated into right thigh with 0.2 cc. of the thick glycerolated suspension. Twelve chicks of the same lot were kept as controls in the same room with the inoculated birds, and allowed to mingle with them.

Results.—Amongst the thirteen inoculated birds, three positive cases developed one of which was of a very severe character, with clinical paralysis and extreme gross and microscopic lesions; the other two presented fairly marked microscopic changes in the peripheral nerves, and were undoubtedly positive although neither obvious clinical symptoms nor gross lesions were present. These birds were sacrificed on the 106th, 125th and 126th days respectively. Three of the thirteen of this group died of an acute infection 7, 8 and 20 days after inoculation—too early for the possible development of paralysis lesions. The incidence may thus be reckoned as three out of ten, or 30 per cent.

In the control group of twelve uninoculated chicks, three died from acute infection on the 18th, 34th and 34th days and may be eliminated from consideration. Of the remaining nine, eight were undoubtedly negative; one only showed a sparse and scattering infiltration of lymphoid cells in one of the bundles of the brachial plexus, and may be classed as a questionably positive case.

In this experiment therefore, which will be repeated on a larger scale, the glycerolated extract of paralysis material proved to be effective in about the same percentage of cases as in the experiments with saline or Tyrode suspension. In the control group there was one doubtful case.

Experiment X.—The following experiment was suggested by the interesting reports of Carrel (5) and White (6) upon the activating influence of dilute arsenious acid in the production of sarcoma in fowl. It was hoped that the introduction of this chemical agent in appropriate concentration in combination with suspensions of nerve tissues from paralysed fowl might induce a multiplication of mononuclear cells and at least reproduce the histopathological features of the disease.

October 21, 1927. Under ether anesthesia[1] and strict asepsis, the sciatic nerves of three healthy White Leghorn cockerels of known paralysis-free stock (Waldo) were exposed. The right sciatic was injected with 0.05 cc. of heavy suspension in Tyrode's (pH 7.4) of ground cord, ganglia, nerve roots and brain from a typical paralysis case, to which had been added dilute arsenious acid to make a final concentration of 1–150,000. The left sciatic was injected in a corresponding place with dilute arsenious acid alone, to determine whether this chemical agent by itself was capable of evoking a cellular reaction.

Three cockerels of the same lot were injected into the right pectoral muscle with 1 cc. of suspension and arsenious acid (final dilution 1–150,000); into the left with arsenious acid alone.

The three chickens which received the intraneural injections were killed after 6, 21 and 28 days. They showed no limping or other evidence of illness during this period. Although there was found, on histological examination, a fairly marked local infiltration of mononuclears which in one of the birds was more marked upon the side injected with suspension and arsenious acid than upon the side injected with arsenious acid alone, the lesions showed no tendency to extend centrally along the nerve trunk, and could perhaps be accounted for by the trauma and introduction of foreign material. The experiment was regarded as inconclusive.

The three birds injected into the pectoral muscle were killed 19, 38 and 50 days after inoculation and were free from symptoms and lesions of paralysis. In the first of these, however, there was found a circumscribed oval tumor mass locally in the muscle between the internal border of the right scapula and the vertebræ. Histologically, this proved to be a sarcoma, composed of large spindle cells, usually bipolar with vesicular nuclei, and surface fibrils; mitoses were numerous. An attempt to transplant fragments with and without the addition of arsenious acid into a cockerel of the same stock, was not successful.

[1] In this and subsequent experiments involving operative procedures, ether anesthesia was used.

The histology of the tumor, its location at a distance from the site of inoculation and the fact that it was found within less than 3 weeks after the injection, make it probable that we were dealing with a spontaneous neoplasm, not evoked by the experimental procedures.

Experiment XI.—November 2, 1927. The previous experiment was repeated on three cockerels of the same stock (Waldo); the right sciatic was injected with suspension of ground sciatic nerve from a paralysed fowl plus arsenious acid to a final dilution of 1–150,000; the left sciatic was injected with the suspension alone.

Results.—Chicken 11–3: 9 days after inoculation a cutaneous nodule 2 mm. in size appeared on the right leg, 2 cm. from the lower border of the incision. This increased in size during the next 4 days; was removed and proved to be a lymphoblastoma. The bird became anemic, lost weight and was killed December 13, 41 days after inoculation.

Autopsy: Large tumor masses were found in thoracic and abdominal cavities, apparently originating in the bursa of Fabricius which was thickened to a cm. or more by tumor tissue, forming an oval mass 3 cm. in length. The center of this was occupied by a sharply sequestrated mass of necrotic tissue. The liver was enormously enlarged, and filled with large and small tumor nodules. Other metastases were present in the kidneys and retroperitoneal cellular tissue. The ovary and oviduct were not involved.

The sciatics, as well as the other portions of the peripheral and central nervous system, showed no significant gross or microscopic lesions.

The development of this extensive visceral lymphomatosis in a chicken inoculated with paralysis material and dilute arsenious acid, is an interesting coincidence in view of the not infrequent association of the two conditions. We do not feel justified, however, in attributing the development of the tumor to the injection, and regard the association as an accidental one.

The two remaining birds of this experiment (Nos. 11–8, 11–2) showed no clinical evidences of paralysis or characteristic microscopic lesions after 48 and 75 days respectively. Two other chickens of the same group (Nos. 11–4, 11–0), inoculated subdurally with 0.15 cc. of the same suspension plus arsenious acid, were negative after 40 and 57 days.

Experiment XII.—November 22, 1927. Three 5 month White Leghorn cockerels were inoculated into the exposed sciatic nerves with suspension of sciatic nerves from a typical paralysis case. Into the right sciatic was injected 0.1 cc. of suspension plus arsenious acid; into the left sciatic, 0.1 cc. of suspension alone.

Results.—Chicken 24–6: Killed after 18 days, having developed fowl-pox. No clinical signs of paralysis. Slight and atypical local lesions at site of injection in right sciatic nerve. Left sciatic negative.

Chicken 29–0: Killed 44 days after inoculation. No clinical or pathological evidence of paralysis.

Chicken 26–2: On February 10 (70 days after inoculation) paralysis suddenly appeared; the right leg was completely helpless, the left leg flexed; general condition good. The chicken was kept under observation until February 20, on which day both legs and wings were paralysed.

Autopsy: Sciatics. Small nodules were found at the site of injection together with diffuse thickening of some of the bundles and disappearance of the normal wrinkling. Characteristic gross changes were found in the brachial plexuses, the vagus and sympathetic nerves and the dorsal root ganglia. There were no significant visceral lesions. Histologically, there were characteristic infiltrations in all blocks examined. But there was no indication that the site of injection was the starting point of the lesions. The nodular swellings noted grossly were due to inflammatory thickening of the perineurium. The cellular infiltrations were more pronounced in the brachial plexuses than in the sciatics.

Experiment XIII.—November 20, 1927. In the following experiment, five 2 day old Rhode Island Red chicks of unknown stock, hatched in the laboratory, were inoculated intramuscularly in the calf or thigh with 0.3 cc. of cord and nerve suspension of a paralysed White Leghorn fowl, plus arsenious acid to a concentration of 1–150,000.

Results.—Three of the five chicks, examined 100, 106 and 180 days after inoculation were negative clinically and pathologically. One chick was lost.

One chicken developed typical paralysis on the 82nd day after inoculation and was found to have characteristic gross and microscopic lesions of ganglia, sciatics and bracheal and lumbosacral plexuses.

In another experiment, weekly injections of dilute arsenious acid (1–150,000) were given over a period of 14 weeks following the inoculation of paralysis suspension. None of the four birds thus treated developed paralysis.

Experiment XIV.—It was thought that the resistance to the hypothetical agent of the disease might be inhibited by a preliminary "blockade" of the reticulo-endothelial system. The report of Roskin (7) indicated that the resistance of birds to the implantation of mammalian neoplasms could be broken down by the intravenous injection of saccharated ferrous oxide.

A group of ten White Leghorn cockerels of known paralysis-free stock was given 5 cc. of saccharated ferrous oxide intravenously; on the following day they were injected by various routes—intracutaneous, intramuscular, intraneural and subdural—with suspension in Tyrode's solution of a typical paralysis case. The chickens were kept under observation for periods of from 58 to 161 days. None of the group showed clinical, gross or microscopic evidence of paralysis, with the exception of one chicken, killed on the 161st day, which showed definite but sparse infiltrations of the sciatic nerves. Preliminary blockades of the reticulo-endothelial system therefore failed to induce an increased susceptibility to inoculation.

DISCUSSION.

The difficulties in the interpretation of these experiments will have become obvious. The insidious development of the disease, the long

latent period between the inoculation and the development of symptoms or frank lesions, the resistance of many individual birds, the occurrence of lesions of every grade of intensity, ranging from insignificant histological infiltrations to massive tumor-like growths, the difficulty of obtaining control material free from lesions—all these factors render the experimental study of the disease laborious and conclusions hazardous. The line between the normal and abnormal as regards the presence of lymphoid tissue about the vessels of the nervous tissue, cannot be sharply drawn, and there is some room for subjective bias. So also with the visceral infiltrations. In the fowl, true lymph glands are not found, but lymphoid tissue is widely disseminated not only through the areolar connective tissue, but within the substance of the viscera. Thus, in routine sections of the heart, collections of lymphoid cells as well as eosinophil myelocytes and polynuclears are found in practically every case. The lymphoid cells are loosely scattered amongst the muscle fibers or collected into follicle-like masses. In the liver also, one finds regularly collections of myeloid or lymphoid elements in the vicinity of the portal spaces, but also definite nodules of lymphoid cells within the substance of the lobules. That such infiltrations may and often do transcend the normal in extent, even to the degree of simulating a pseudoleucemia cannot be denied, but the transition between the normal and the pathological is not defined.

In spite of these various difficulties, one general conclusion seems firmly established by our experiments and that is the significantly higher incidence of typical neurolymphomatous lesions in the inoculated birds as compared with the controls. This result was consistently obtained in all the experiments in which unfiltered suspension of nerve tissue from paralysed fowl was used. Taking only the unmistakable and characteristic positive cases, we find in 89 inoculated birds, 24 positive cases, an incidence of 26+ per cent; while in the control groups comprising in all 104 birds kept under comparable conditions, only 7 cases of paralysis developed—an incidence of less than 7 per cent. This proportion was approximately maintained in each of the several experiments—a fact which lends added significance to the data.

We feel justified therefore in drawing the conclusion that the

inoculation of the material from paralysed fowl definitely favored the subsequent development of the disease.

The mere fact that the disease may be experimentally transmitted to a certain proportion of inoculated chickens hardly warrants one in drawing inferences as to the nature of the causative agent or agents, and speculation at this time seems unprofitable. The definitely neoplastic character of the infiltrating tissue in some cases brings the disease in line with the transplantable sarcomata of chickens. In Experiment XVIII, evidence was secured that the active agent may survive glycerolation; and several other considerations make it unlikely that transmission is accomplished by the implantation of viable cells. The long period elapsing between inoculation and the development of characteristic lesions is against this possibility, as is the fact that there is no local accumulation of cells at the site of inoculation. In the limited number of experiments we have not been able to demonstrate the filtrability of the agent but further work is needed to reach a conclusion on this point.

Although the evidence, epidemiological and experimental, points to an infective agent, we are totally ignorant as to its nature, and as to the manner in which the disease is conveyed under natural conditions. It is in the hope of stimulating further research in this interesting and important problem that these experiments are submitted.

CONCLUSIONS.

1. Inoculation of suspensions of brain, cord, ganglia or nerves of chickens with neurolymphomatous lesions, into newly hatched chicks, is followed by the development of typical lesions in approximately 25 per cent of cases.

2. In control chickens kept under laboratory conditions the incidence of the disease is about 7 per cent.

3. The disease does not become manifest until at least 2 months after inoculation; symptoms may not appear until after 4 months.

4. The active agent is not destroyed by 50 per cent glycerol in 9 days at ice box temperature.

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CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD OF RABBITS.

IV. Influence of Light Environment on Normal Rabbits.

By WADE H. BROWN, M.D.
With the Collaboration of Marion Howard.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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By a systematic study of the blood of normal rabbits (1), it was found that both the absolute and relative amounts of calcium and inorganic phosphorus exhibited periodic variations and that, among animals living in the laboratory, there was evidence of a tendency toward a progressive increase in calcium and of a decided decrease in inorganic phosphorus of uncertain duration and extent. It appeared that, while a number of factors may have contributed to the production of the variations noted, one of the chief causes was the occurrence of changes in the light environment.

It is, of course, well known that ultra-violet radiations of short wave-length (3100 Ångström units or less) are capable of exerting a profound influence on the inorganic phosphorus content of the blood and hence on the calcium-phosphorus equilibrium. In the present instance, however, it was evident that the variations found among animals living in the laboratory could not be attributed to the action of ultra-violet light of short wave-length as the light received by these animals was filtered through glass.

From previous experiments (2), it had been found that rabbits living under similar conditions showed periodic variations in the weights of organs, or organic constitution, and in their reaction or susceptibility to disease, and that these conditions were subject to the influence of a light environment which included no ultra-violet of short wave-length. It seemed probable, therefore, that blood calcium and inorganic phosphorus might be influenced by similar

conditions. This assumption was supported by the fact that Mayerson, Gunther, and Laurens (3) found that, in dogs, calcium and phosphorus were affected by a change from room light to darkness or *vice versa*, and they emphasized the fact that the results obtained by them were similar in character to effects produced by irradiation with the carbon arc.

In studying the influence of light environment on the calcium and inorganic phosphorus content of the blood, our chief interest was in effects of conditions involving light of long wave-length and a minimum of radiant energy as compared with the intensity of illumination, that is, in conditions as far removed from the ultra-violet end of the spectrum as possible. The experiments carried out were based on a comparison of certain reactive phenomena presented by animals living under the usual laboratory conditions, in total darkness, or in an environment of neon light. In addition to calcium and inorganic phosphorus, parallel observations were made on the effects of the conditions mentioned on nutrition and growth, the growth of hair, on organic constitution, and on the reaction to disease. The results for the first three phases of the investigation have been reported (4); those for calcium and inorganic phosphorus will be presented in the present paper.

Material and Methods.

The animals used for the experiments to be reported were the same as those used for the study of the influence of light on nutrition and growth, the growth of hair, and organic constitution. The conditions of the experiments were given in detail in the first of these papers (4) but may be repeated in part for the sake of clarity.

The experiments to be reported represent parallel observations on rabbits living under conditions which differed only with respect to the light environment. One group of animals, which for convenience may be designated as the controls, was kept in a well lighted (sunlight), well ventilated room with a southern exposure. The room was heated during cold weather and an effort was made to maintain a temperature of 60–70°F. (thermostatic regulation). These animals are designated as controls merely because they represented the usual as contrasted with an unusual condition.

A second group of rabbits was kept in a room from which all light was excluded. Other living conditions were the same as those of the first group. During the course of the experiments, these animals received some light from two sources. For a brief period each day, a 30 watt Mazda lamp was used in this room for clean-

ing cages, feeding, and making the necessary observations. From time to time, the animals were also brought into the laboratory for bleeding and were thus exposed to diffuse filtered sunlight for a period which rarely exceeded an hour per week.

A third group of animals, the light group, was kept in a room similar to the others, with sunlight excluded. This room was lighted continuously by 20 standard neon tubular lights (crown glass), 6 feet in length and 5/8 inch in diameter. The tubes were mounted in an upright position on a rectangular frame in the center of the room, parallel with and facing the cages at a distance of approximately 3½ feet. The lights were operated on a current of 25 milliamperes at 8000 volts to a bank of 3 lamps in series.

The terms control, light, and dark are used with reference to the three groups of animals in a purely descriptive sense. Each group was intended to supply information concerning the biological action of a particular kind of light environment and each served as a control to the others.

As is well known, the light produced by neon Geissler tubes varies from a rose-red to a bluish pink color, depending upon the condition and the operation of the tube. The heat produced is very slight. The rays transmitted by crown glass are concentrated in two regions with faint bands intervening. The shortest rays lie between 3370 and 3620 Ångström units. The strongest of these are in the region of 3460 and 3480. The greater part of the light produced is of very long wave-length and is composed of rays from about 5800 to the lower limit of registration at 7600 Ångströms. The vertical illumination at the front of the cages varied between 21 or 23 foot candles at the ends of the rack and 54 at the center with a mean of 34.3 foot candles. No attempt was made to measure the energy delivered by this equipment as it was perfectly obvious that such measurements would serve no particular purpose in the present instance.

We wish also to emphasize the fact that the animals used in these experiments were given no special preparation to facilitate the action of light such as clipping or shaving of hair.

Observations were made on 3 groups of normal male rabbits living under the conditions described. At the beginning of the experiments, the animals were 8 to 10 months old; they were sexually mature but had not attained their full growth. In each case, the animals for a given experiment were assembled and divided into 3 comparable subgroups according to type or breed, age, and weight, but without any knowledge of the calcium or phosphorus content of the blood. All animals were caged separately and fed a uniform diet of hay, oats, and cabbage.

Group I contained 15 albino rabbits, Group II, 15 black rabbits, and Group III, 15 gray, brown, or black rabbits. In each case there were 5 animals exposed to the neon light, 5 in the dark, and 5 received diffuse, filtered sunlight of varying intensity and duration.

The animals of Groups I and II were placed under observation Oct. 1, 1926,

and kept under control conditions until Oct. 22. Determinations of calcium and inorganic phosphorus were begun Oct. 8 and continued at weekly or biweekly intervals until May 18, 1927. 323 calcium and 321 phosphorus determinations were made on Group I and 329 and 327 on Group II.

The animals of Group III were also placed under observation Oct. 1, 1926, and were put into their experimental quarters at that time, but no blood analyses were made until Dec. 1. The blood of these animals was examined at irregular intervals and on Feb. 8, 1927, the light and dark divisions of the group were interchanged so that animals that had been living in an environment of neon light were placed in the dark and *vice versa*. This condition was maintained until May 4, when the two groups were restored to their original environmental conditions and observations were continued until June 8, 1927.

For this group of animals there are 3 sets of results: first, a series of 20 blood analyses on each of the 3 subdivisions, most of which were made during the last month of a 4 months period of continuous exposure to neon light or confinement in the dark with corresponding observations on the controls; second, a series of 45 blood analyses covering a period of nearly 3 months during which the light environment of the experimental divisions was reversed; and a series of 20 analyses covering a final period of approximately 1 month during which the animals were restored to their original environmental conditions. The total number of calcium and of phosphorus determinations made on the 3 groups of animals was 907 and 903 respectively.

The methods employed in making determinations of calcium and inorganic phosphorus were described in detail in the first paper of this series (5).

The results for calcium, inorganic phosphorus, and the ratio of calcium to inorganic phosphorus are recorded in Tables I to IV as group means for consecutive determinations. The values for calcium and phosphorus are given in mg. per 100 cc. of serum.

The general trend of the variations observed and the relative magnitude of the values obtained are shown graphically in Figs. 1 to 18. These curves were obtained by smoothing the group means as given in Tables I to IV by the formula $\frac{A + 2B + C}{4}$ and converting the smoothed values into percentage deviations from the mean values obtained for a large series of determinations on normal rabbits (5).

RESULTS.

The results of the experiments are summarized in Tables I to IV and Figs. 1 to 18.

TABLE I.

Calcium. Group Means for Consecutive Determinations.

Date	Group I			Group II		
	Control	Light	Dark	Control	Light	Dark
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Oct. 8	13.66	13.8		13.8	13.7	14.2
" 15	13.15	15.1	14.3	13.3	14.0	13.8
" 21	15.79	14.0	15.0	15.0	13.6	16.0
" 29	15.43	13.9	15.3	14.9	13.1	15.0
Nov. 5	16.50	16.5	15.2	15.6	16.2	16.5
" 12	13.68	14.2	14.1	14.2	13.9	15.0
Nov. 19	15.16	15.0	15.3	15.2	14.6	15.9
" 26	14.62	14.7	14.8	14.7	14.0	14.9
Dec. 3	15.35	15.3	15.7	15.2	14.8	15.9
" 10	15.51	15.5	15.7	15.5	15.6	15.5
" 17	16.12	15.5	15.7	14.8	15.5	15.8
" 31	15.77	16.0	16.5	16.1	15.4	15.5
Jan. 14	15.40	15.2	16.3	15.4	15.5	17.0
" 28	16.68	16.2	15.8	17.1	16.3	16.2
Feb. 11	15.20	15.2	16.1	15.3	15.0	16.1
" 25	15.56	15.8	16.1	15.6	14.6	16.2
Mar. 11	16.04	15.8	16.1	15.5	15.3	15.8
Mar. 25	15.84	15.4	15.0	15.4	15.2	15.9
Apr. 8	15.23	15.5	15.3	15.3	14.8	16.1
" 22	16.51	15.6	15.1	15.1	14.5	15.6
May 4	15.43	15.0	14.5	15.5	15.1	14.6
" 18	17.18	17.5	17.0	18.3	16.4	17.1

TABLE II.

Inorganic Phosphorus. Group Means for Consecutive Determinations.

Date	Group I			Group II		
	Control	Light	Dark	Control	Light	Dark
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Oct. 8	4.41	5.48		5.95	5.80	4.09
" 15	4.47	3.79	4.17	4.63	3.48	4.73
" 21	3.39	4.22	3.98	4.91	4.55	5.19
" 29	4.00	4.43	4.24	4.31	4.50	4.76
Nov. 5	4.67	4.32	4.22	4.87	5.05	4.79
" 12	4.73	5.29	4.63	5.31	5.39	4.73
" 19	4.90	4.27	4.63	5.06	4.78	4.49
" 26	4.39	4.71	4.56	5.01	4.86	4.65
Dec. 3	4.61	4.70	3.99	4.43	4.19	3.98
Dec. 10	4.18	4.24	4.38	4.33	4.48	4.21
" 17	3.61	4.23	4.38	4.59	3.91	4.09
" 31	4.40	3.56	4.39	4.72	4.12	4.25
Jan. 14	4.19	4.48	4.02	4.50	4.55	3.91
" 28	3.90	3.55	3.66	3.98	3.61	3.90
Feb. 11	4.14	4.01	4.06	4.15	4.35	3.99
Feb. 25	4.21	4.05	3.76	4.08	3.98	3.97
Mar. 11	3.86	3.41	3.39	3.92	3.50	3.52
" 25	3.81	3.67	3.65	3.88	3.87	3.70
Apr. 8	3.95	3.62	3.17	3.56	3.69	3.41
" 22	3.56	3.50	3.10	3.32	3.25	2.85
May 4	3.39	3.37	3.24	3.13	3.51	3.20
" 18	3.93	3.88	3.85	3.96	3.88	3.75

TABLE III.

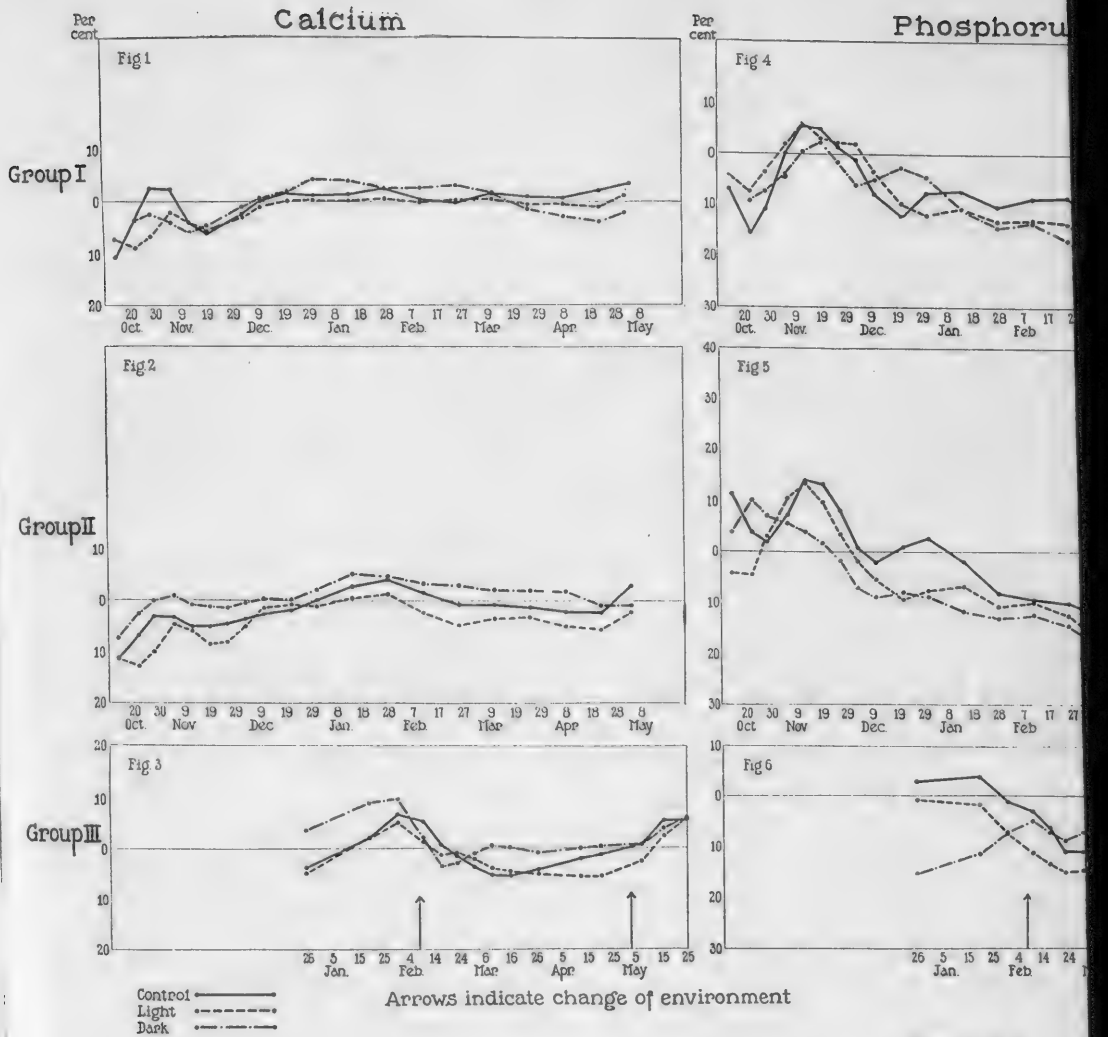
Calcium-Phosphorus Ratio. Group Means for Consecutive Determinations.

Date	Group I			Group II		
	Control	Light	Dark	Control	Light	Dark
Oct. 8	3.11	2.52		2.32	2.36	3.47
" 15	2.95	3.98	3.43	2.87	4.02	2.92
" 21	4.66	3.32	3.77	3.06	2.99	3.08
" 29	3.85	3.14	3.61	3.46	2.91	3.15
Nov. 5	3.73	3.82	3.60	3.20	3.21	3.45
" 12	2.90	2.68	3.05	2.67	2.58	3.17
" 19	3.10	3.51	3.31	3.00	3.05	3.54
" 26	3.33	3.12	3.25	2.93	2.88	3.20
Dec. 3	3.34	3.26	3.94	3.39	3.53	4.00
" 10	3.71	3.66	3.59	3.58	3.48	3.68
" 17	4.46	3.66	3.59	3.22	3.96	3.86
" 31	3.59	4.49	3.76	3.41	3.74	3.65
Jan. 14	3.68	3.39	4.06	3.42	3.46	4.35
" 28	4.28	4.56	4.32	4.30	4.52	4.15
Feb. 11	3.67	3.79	3.97	3.69	3.45	4.04
" 25	3.71	3.93	4.28	3.82	3.67	4.08
Mar. 11	4.15	4.63	4.75	3.95	4.37	4.49
" 25	4.15	4.20	4.11	3.97	3.93	4.30
Apr. 8	3.85	4.28	4.83	4.30	4.01	4.72
" 22	4.64	4.46	4.87	4.55	4.46	5.47
May 4	4.54	4.45	4.48	4.95	4.30	4.56
" 18	4.38	4.51	4.42	4.62	4.23	4.56

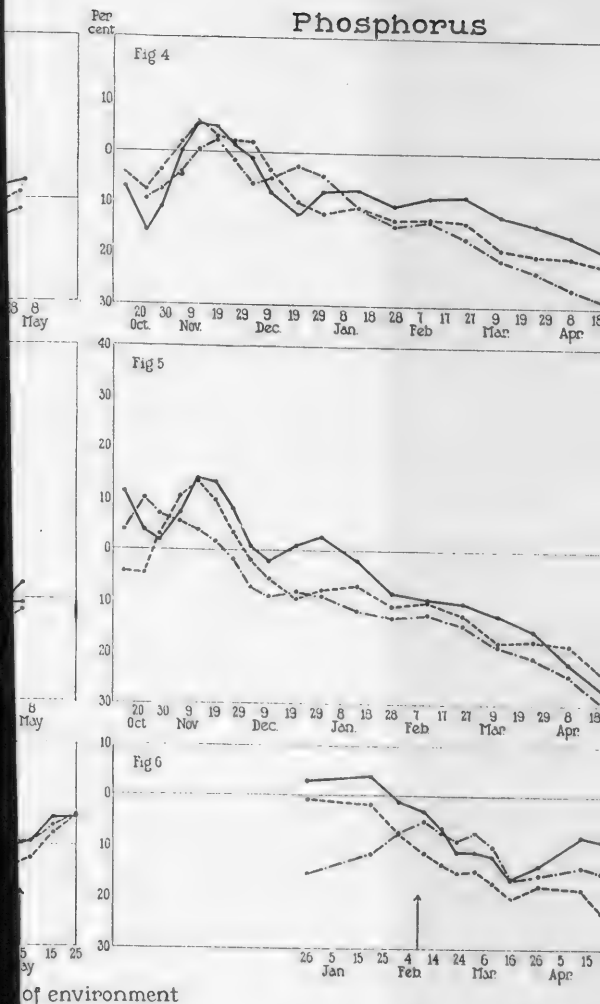
TABLE IV.

Group III. Group Means for Consecutive Determinations.

Date	Calcium			Phosphorus			Calcium-phosphorus ratio		
	Control	Light	Dark	Control	Light	Dark	Control	Light	Dark
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*			
Dec. 1	13.9	14.0	15.0	3.83	3.98	3.71	3.64	3.51	4.05
Jan. 5	15.3	14.7	16.6	5.03	4.81	3.77	3.04	3.05	4.41
" 19	15.6	15.9	16.5	4.66	4.33	4.04	3.34	3.67	4.08
Feb. 4	17.2	17.2	18.4	4.38	4.27	4.13	3.93	4.04	4.44
Feb. 11	16.6	15.3	15.2	4.41	3.85	4.46	3.72	3.97	3.42
" 16	15.4	15.4	14.9	4.32	4.07	4.10	3.55	3.77	3.63
" 28	15.6	15.5	15.2	3.85	3.66	4.10	4.04	4.25	3.70
Mar. 2	15.0	15.5	15.3	4.07	3.95	4.19	3.67	3.92	3.65
" 9	14.7	14.6	15.9	4.10	3.84	4.28	3.58	3.80	3.71
" 16	14.8	15.2	15.6	3.64	3.37	3.53	4.06	4.51	4.41
" 23	14.8	14.5	15.4	3.72	3.85	3.82	3.96	3.77	4.03
Apr. 6	15.5	15.0	15.5	4.53	3.81	4.11	3.41	3.94	3.77
May 4	15.4	14.4	15.9	3.87	3.28	3.53	3.98	4.40	4.51
May 6	15.2	15.1	15.3	4.21	3.59	4.17	3.62	4.20	3.66
" 11	17.0	16.1	16.3	3.90	3.50	3.83	4.35	4.60	4.27
" 25	16.6	16.6	16.9	3.76	3.71	3.72	4.41	4.47	4.54
June 8	15.8	16.7	16.0	3.61	3.37	3.56	4.36	4.95	4.48


Calcium
Phosphoru
Per cent
Fig 1
Fig 2
Fig 3
Fig 4
Fig 5
Fig 6
Group I
Group II
Group III
Oct.
Nov.
Dec.
Jan.
Feb.
Mar
Apr.
May
Control
Light
Dark
Arrows indicate change of environment

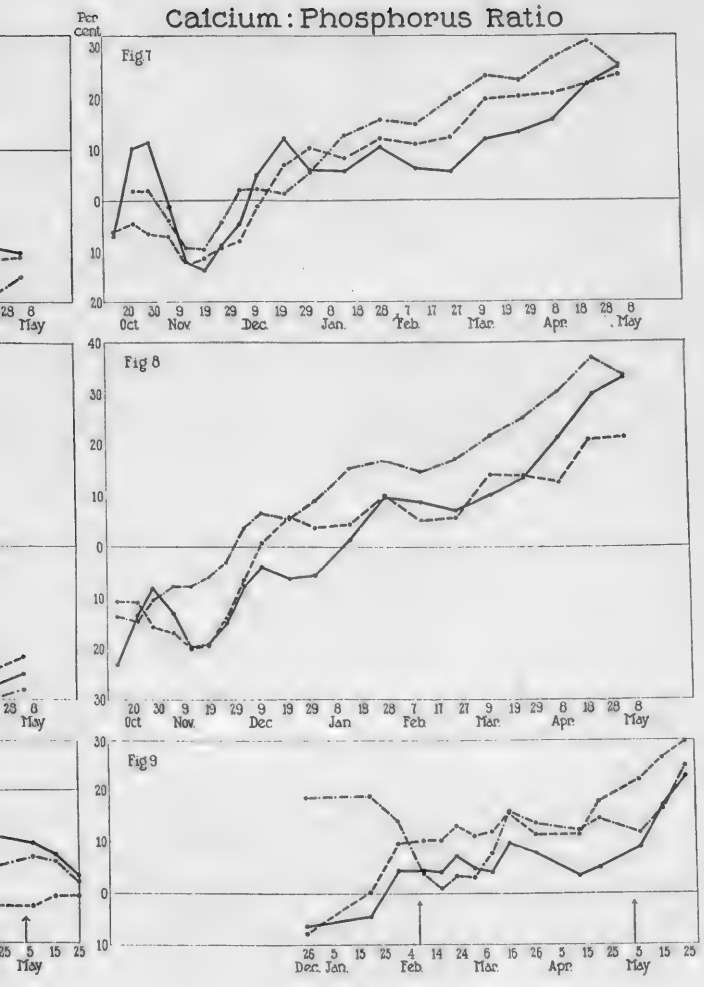
FIGS. 1 to 9.

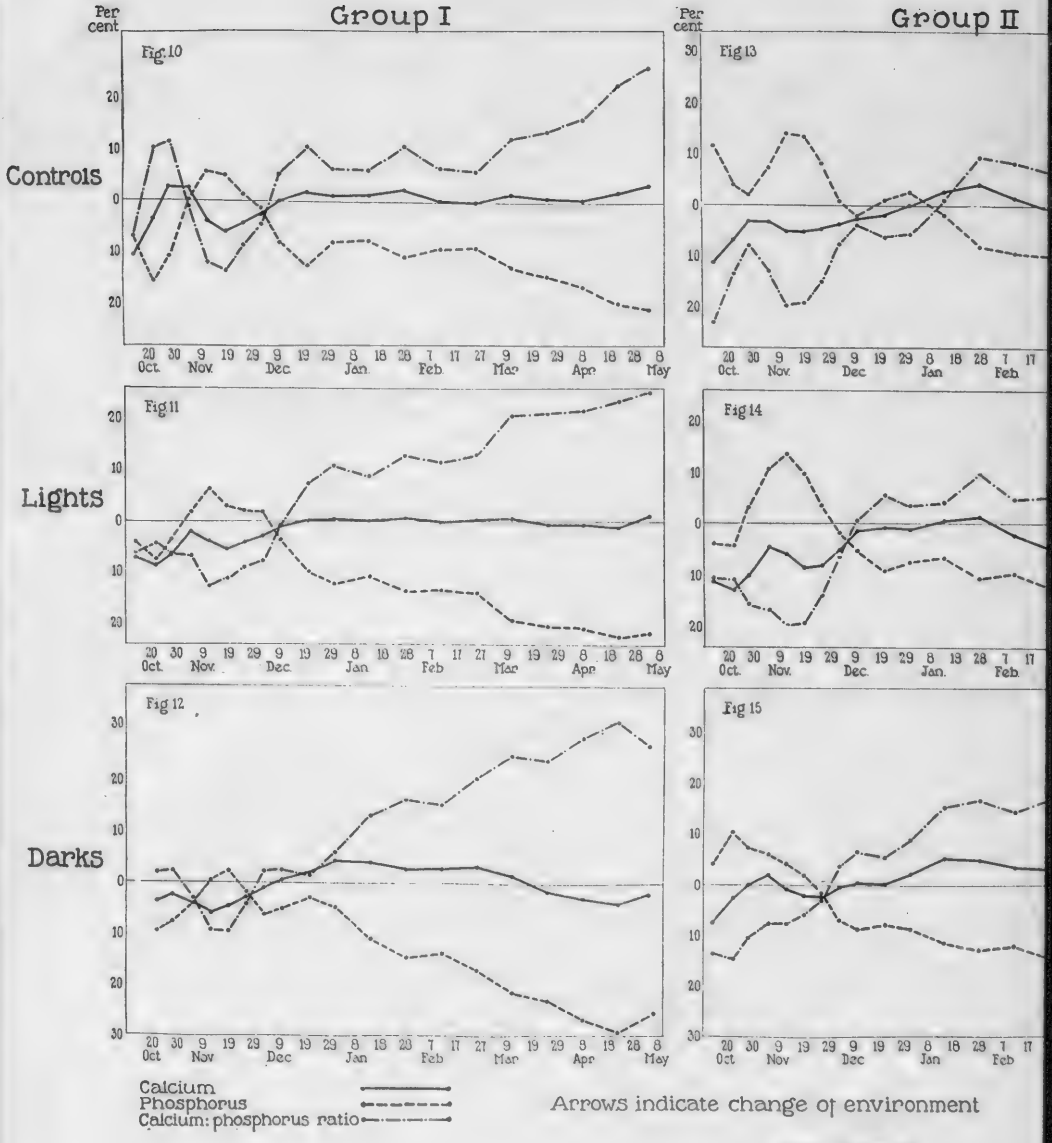

Phosphorus
Per cent
Fig 4
Fig 5
Fig 6
20 30 9 19 29 9 19 29 8 18 28 7 17 27 9 19 29 8
Oct. Nov. Dec. Jan. Feb Mar Apr
8 May
26 5 15 25 4 14 24 6 16 26 5 15
Jan Feb Mar Apr

of environment

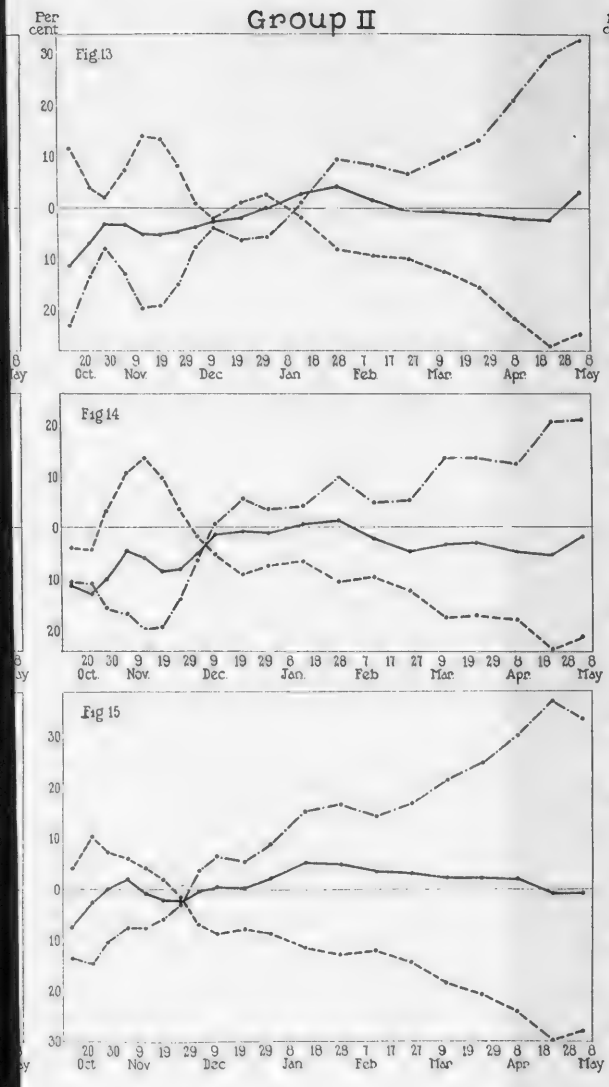
FIGS. 1 to 9.

Calcium : Phosphorus Ratio




Group I
Group II
Per cent
Controls
Lights
Darks
Fig 10
Fig 11
Fig 12
Fig 13
Fig 14
Fig 15
20 30 9 19 29 9 19 29 8 18 28 7 17 27 9 19 29 8 18 28 8
Oct. Nov. Dec. Jan. Feb. Mar Apr May
20 30 9 19 29 9 19 29 8 18 28 7 17
Oct. Nov. Dec. Jan. Feb.
Calcium
Phosphorus
Calcium: phosphorus ratio
Arrows indicate change of environment

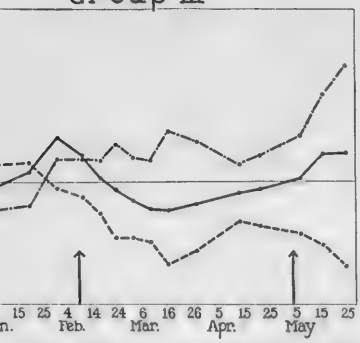
FIGS. 10 to 18.

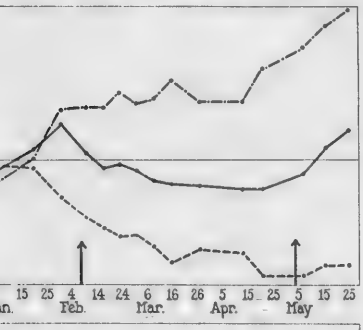


licate change of environment

FIGS. 10 to 18.

Group III


15 25 4 14 24 6 16 26 5 15 25 5 15 25
n. Feb. Mar. Apr. May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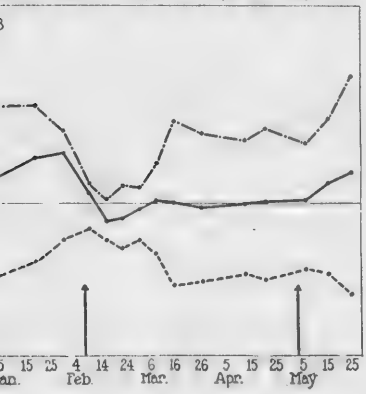
n. Feb. Mar. Apr. May


15 25 4 14 24 6 16 26 5 15 25 5 15 25
an. Feb. Mar. Apr. May

DISCUSSION AND CONCLUSIONS.

Analysis of the results will be facilitated if we consider certain features of the reaction shown by the smoothed curves in Figs. 1 to 18 before attempting to make a comparison of actual values.

An examination of the curves for calcium and inorganic phosphorus of Groups I and II (Figs. 1, 2, 4, 5) shows that the changes found in the blood of these animals are divisible into several phases. First, there was a period during which wide variations occurred from one examination to the next. This condition lasted from the beginning of the experiments until about Nov. 19 or 26, or for a period of 7 to 9 weeks. During the next month, the changes that occurred were more gradual and assumed a more orderly progression in a given direction. In general, calcium increased and inorganic phosphorus decreased. This was followed by a third period of 6 to 8 weeks during which there was comparatively little change of level or, in the case of calcium, there was a gradual reversal of the direction of the curves. Finally, at the end of 4 to 5 months, the magnitude of the variation increased again with a decrease in both calcium and phosphorus which changed to an increase as the experiments were concluded.

It will be noted that variations of this general type were shown by control, light, and dark animals. There were, however, distinct differences in the magnitude and direction of the variations that occurred as well as in the time relations, while the results for Group I were not identical with those for Group II.

The problem presented is to determine whether these variations in the calcium and phosphorus content of the blood were influenced to an appreciable extent by light environment and, if possible, the nature of the influence exerted by a particular environmental condition.

The results obtained for control animals during the first 7 to 9 weeks are clearly indicative of a profound disturbance of the calcium-phosphorus equilibrium. As was pointed out in a previous paper (1), a change of this kind is almost invariably produced by a change from outdoor to indoor life. Mayerson, Gunther, and Laurens (3) found that similar disturbances of the calcium-phosphorus equilibrium were produced in dogs by a change from room light to darkness or *vice versa*, or by irradiation with the carbon arc. It seems, therefore, that the first series of variations which occurred in the blood of control animals

may be regarded as a reaction to the change in the mode of living in which the light environment, and the deprivation of ultra-violet in particular, was an important factor.

Animals of the light and dark divisions were subject to the same influences as the controls for a period of 3 weeks, and they show evidence of the occurrence of changes similar to but not identical with those of corresponding controls. Among the white animals of Group I, the increase in calcium during this period was distinctly less. This is particularly true of the dark division which showed a decrease rather than an increase during the time that the values for control animals were at their maximum (Fig. 1).

The results for the black animals of Group II were different (Fig. 2). The increase in calcium for controls was less than that of Group I, but the results for the dark division virtually paralleled those for the controls at a slightly higher level, while the change in the light division was essentially the same as for Group I.

The results for inorganic phosphorus show a similar situation (Figs. 4 and 5). There was first, a decrease, and then a marked increase and a second decrease in which the animals of the light division followed the controls fairly closely. Among the albino rabbits in the dark, the reaction followed a similar course but the increase was less, as in the case of the calcium, while the black animals (Group II) showed a marked decrease in phosphorus during the period of increase in the control and light divisions.

One effect of these changes on the calcium-phosphorus equilibrium is shown in Figs. 7 and 8. In the case of Group I, it will be seen that the differences produced in the ratio of calcium to inorganic phosphorus during this first period of adjustment or accommodation to indoor life and to exposure to neon light or confinement in the dark are both qualitative and quantitative.

The effect on the controls was a marked increase in the ratio, followed by an even greater decrease, while the ratios for light and dark animals remained virtually constant during the period of increase, and the subsequent decrease was less than that shown by the controls. Results of a similar character were obtained for Group II, but in this case, after a slight drop in the calcium-phosphorus ratio, the animals in the dark showed a marked rise which was maintained during the

period of secondary decrease shown by control and light animals. This increase in the calcium-phosphorus ratio is, of course, influenced more by the reduction in inorganic phosphorus than by the increase in calcium which occurred at this time.

From a comparison of the results for this period, it seems that the most obvious effect of exposure to neon light or complete exclusion of light was to diminish the reaction of accommodation to indoor life as shown by control animals. While the actual influence exerted by the experimental conditions is more or less obscured by the reaction of accommodation, it seems that, in general, exclusion of light tended to produce a reduction in both calcium and inorganic phosphorus and that the influence of neon light was to depress the one and to increase the other. The effect of exclusion of light was greater than that of light of long wave-length. It also appears that the black animals were more susceptible to the influence of these conditions than the albinos; this is particularly true of the influence on inorganic phosphorus, but there is a suggestion that, in the case of calcium, the color relation may have been reversed.

During the second period of the experiment, the calcium in the blood of control animals increased; in Group I, it reached a maximum toward the end of December, but in Group II the rise continued until the end of January (Figs. 1 and 2). There was a similar increase in calcium among light and dark animals with the establishment of a definite order of magnitude, the animals in the dark giving the highest values, those in the light the lowest, and the controls occupying an intermediate position. The differences in level were comparatively slight, but were greater in the case of black than of white animals which is of interest in comparison with the results for Group III.

No blood analyses were made on the animals of Group III until Dec. 1, or until they had been under observation for 2 months. The results begin, therefore, with the period corresponding with the period of increasing calcium in Groups I and II, and we find precisely the same relations in Group III (Fig. 3) but with an even greater difference between the control and light animals on the one hand and dark animals on the other.

As calcium increased in Groups I and II, inorganic phosphorus decreased, and, in Group II, the decrease shown by animals of both

the light and dark divisions was greater than that of the controls. Among the control animals, the decrease in inorganic phosphorus was interrupted by a clearly defined secondary rise and a little later there was a slight increase among the animals of the light division, while in the dark division there was merely a reduction in the rate of decrease.

The relative order of magnitude of values for inorganic phosphorus was not exactly the reverse of that for calcium, but the lowest values were given by animals in the dark and the highest by the controls with the light division in the intermediate position.

The reaction of the white animals of Group I was somewhat different. In the first place, the high values reached during the preliminary period of adjustment were maintained for a longer time and the subsequent decrease in phosphorus was more gradual. This was particularly true of animals in the light division which maintained a higher level during this period than the controls. The reaction of animals in the dark was also peculiar in that they showed a secondary rise in inorganic phosphorus, the maximum of which coincided with the minimum of the controls, so that during the second half of the period, the relative position of these animals changed from lowest to highest.

The results for Group III (Fig. 6) agree with those for Group II in so far as the relative amounts of inorganic phosphorus in the blood of the 3 classes of animals are concerned and with even greater quantitative differences. But, in Group III, phosphorus rose in the blood of animals in the dark, while it decreased in the control and light divisions as in Group I.

Considering the 3 groups of animals, it is again evident that the general trend of the curves for calcium and inorganic phosphorus were essentially the same irrespective of differences in environmental conditions. There were, however, differences in the order of magnitude of the values obtained, and in the case of phosphorus, definite differences in the direction of the curves at certain times. In general, the evidence indicates that complete exclusion of light tended to diminish the inorganic phosphorus content of the blood and to increase the calcium especially in animals of a dark color, but was less effective in the case of albino rabbits. There is also evidence that neon light exerted a similar influence on the phosphorus content of the blood, but its action differed from that of complete darkness in that the

depression of the phosphorus was less while the calcium content of the blood was diminished and not increased.

The effect of these changes on the calcium-phosphorus equilibrium (Figs. 7, 8, and 9 and Tables III and IV) was to increase the ratio of calcium to inorganic phosphorus of all classes of animals in Groups I and II and of the control and light divisions of Group III. The dark division of the third group was first increased to a level far above that of control and light animals, and then diminished.

The relative magnitude of the change varied in different groups of animals. In Group I (albinos), the controls gave the highest value for this period, the light division came next, and the dark animals were lowest despite the fact that during the first half of the period the ratio was highest in these animals. In Groups II and III (dark colored animals) the order of magnitude was reversed with a ratio of calcium to phosphorus that was much higher in dark than in either the light or control animals, while the values for the light divisions were much higher than those for the controls.

Following the period of increasing calcium and decreasing phosphorus, the calcium level in control animals of Group I remained high for about 3 months. At the beginning of this period inorganic phosphorus increased slightly and then remained comparatively constant for about 2 months.

The values obtained for calcium in light and dark animals differed from those of the controls in that they showed less fluctuation and were at slightly lower and higher levels. There was a stabilization of inorganic phosphorus in the light animals during the latter part of the period, as in the controls, and a suggestion of a similar condition in the dark division, but on the whole, there was a continuation of the downward movement which was more marked in dark than in light animals.

The results for Group II differed somewhat from those for Group I. In the first place, the period of increasing calcium was more prolonged and this was followed by a definite decrease which was associated with a marked decrease in inorganic phosphorus. Light and dark animals reacted in a similar manner. There was, however, less reduction in the calcium of dark animals, while the light division showed a slight rise following an initial reduction of considerable duration and extent.

The relative magnitude of the three values was the same, however, as for Group I.

Following the increase of inorganic phosphorus in control animals of Group II (December) there was a marked decrease over a period of about 1 month. The decrease then continued at a rate virtually parallel with that of animals in the dark, but at a distinctly higher level. The animals in the light also showed a reduction in inorganic phosphorus, but in this case, the decrease was interrupted by brief periods of slight increase or stabilization.

During the last 2 months of the experiments, there were several changes in the relative magnitude of values. For some time, animals in the dark had shown higher values for calcium than either the controls or those in the light, but among the dark animals of Group I, calcium decreased until it was at a distinctly lower level than in either of the other divisions. In Group II the reduction occurred later, and when the experiments were concluded, the calcium level for dark animals was still slightly higher than that of animals in the light but lower than the controls.

Inorganic phosphorus decreased in control and dark animals of both groups with no material change of relation, but the decrease was more marked in Group II than in Group I. Among the animals in the light, the decrease was less; the difference between the light and dark divisions was increased, while in Group II the phosphorus level was higher than that of the controls with an evident tendency in the same direction in Group I.

As the experiments were concluded, all values were increasing again with the exception of the calcium in the dark division of Group II and the phosphorus of control animals of Group I.

The entire series of changes described above is presented in Figs. 10 to 15 from the point of view of the relation between the changes that occurred in calcium to those in inorganic phosphorus and their effect on the calcium-phosphorus equilibrium as indicated by the ratio between the two substances. We cannot attempt a detailed discussion of these curves, but it will be seen that while the relations presented assume the same general form in all cases, the exact relations are different as indicated by the spacial relations and directions of lines. The difference between the equilibrium shown by control and light animals is, on the whole, less than that shown by dark animals in comparison with

other groups. There are, in fact, 3 types of curves of which the one presented by animals in the dark is most distinctive. A comparison of results from this point of view shows that differences in absolute amounts of calcium or phosphorus, which might appear to be insignificant, may produce a very definite difference in the equilibrium between the two substances.

Another feature of the results to be considered is the effect of a change of environment on animals that have lived for several months in a fixed environment of neon light or in darkness as indicated by the results obtained for Group III (Figs. 3, 6, 9, and 16 to 18). When the first change was made (Feb. 8), calcium had reached a high level in all classes of animals, while inorganic phosphorus was decreasing in animals of the control and light divisions and, in the dark, increasing from an exceptionally low level.

Following the change of environment, there was a marked decrease in the calcium of the animals transferred from dark to light and a smaller decrease among those transferred from light to dark, but the controls also showed a decrease which continued for some time, while there was a slight and transient secondary rise among the animals that had been placed in the dark and a more decided and more prolonged rise among those that had been transferred from the dark to the light. This increase in calcium was maintained while the calcium of animals placed in the dark decreased to a level below the controls. Meantime, there was a gradual increase of calcium in control animals. After a brief period of adjustment there was thus a return to the relation that existed between light and dark animals at the time of the change in environmental conditions with the reestablishment of fairly stable levels, while the controls showed changes which were not reflected to an appreciable extent by either the light or dark divisions.

The immediate effect of the change of environment on the phosphorus is uncertain. There may have been an accentuation of the decrease in inorganic phosphorus of animals placed in the dark and a corresponding tendency toward a sustained elevation among those transferred to the light. At any rate, there was a change in the relative levels of the two groups of animals, and after a further decrease in inorganic phosphorus, the animals placed in the light showed an increase while the decrease continued among those in the dark.

When the second change of environment was made (May 4), both calcium and phosphorus were at lower levels than at the time of the first change. Calcium increased in both the light and dark animals, while inorganic phosphorus diminished in the dark group and increased slightly in the light, but it is not certain that these changes in the chemical composition of the blood can be regarded as an effect of the change in environment as the period of observation was too short to afford an opportunity for a definite determination of results.

If we compare the effect of this series of changes on the calcium-phosphorus equilibrium, as shown in Figs. 16 to 18, with conditions presented by the animals of Groups I and II (Figs. 10 to 15), it will be seen that, following the initial disturbance of equilibrium, the curves for both light and dark animals were more analogous to those of the light divisions of other groups than to the dark divisions. This is shown by the tendency to maintain a comparatively constant ratio. There is, however, a suggestion of the persistence of a dark effect in the high calcium-phosphorus ratio of dark animals while the tendency of this value to decrease rather than to increase is indicative of a light effect. It is to be noted that this decrease in ratio values occurred despite the persistence of a high calcium level, which may be regarded as a dark effect, and was due to an increase in inorganic phosphorus which is indicative of a light effect. The converse is true for animals that were transferred from a light to a dark environment so that, comparing the spacial relations of the three values shown in these figures with corresponding curves for Groups I and II, it appears that, despite the persistence of certain conditions which may be attributed to preexisting environmental influences, the change of environment reversed the general relations of the curves.

Thus far, the discussion has been confined to a consideration of the trend of the curves for calcium and inorganic phosphorus and such differences in results as are shown by comparisons of smoothed curves. It is evident that all classes of animals showed actual variations in calcium and inorganic phosphorus comparable with the periodic and progressive variations reported in a previous paper (1), so that the evidence bearing on this phase of the results need not be considered further. In addition, there is definite evidence of quantitative and qualitative differences between the results obtained for animals living under different environmental conditions as well as for animals

of different breeds or color. The differences shown appear to be consistent and, while it is highly improbable that, by chance alone, variations of the same character would be obtained in all of the experiments, the differences indicated are, for the most part, comparatively small and the question arises as to whether they are sufficiently large to be regarded as significant. In order to answer this question, a quantitative comparison of results is necessary.

A general idea of the situation presented may be obtained by a comparison of the mean values for all observations on a given lot of animals, disregarding variations in the magnitude and direction of changes that occurred. A comparison of this kind constitutes the most severe test that can be applied since the occurrence of variations in one direction and then in the other might obliterate any difference between mean values or obscure the effect of an influence which tended to produce a variation in a given direction. On this basis, the results obtained for calcium, inorganic phosphorus, and for the calcium-phosphorus ratio are as follows:

Calcium.

	Group I	Group II	Group III
Control	15.3 ± .09	15.4 ± .09	15.5 ± .10
Light	15.3 ± .07	14.9 ± .07	15.4 ± .08
Dark	15.5 ± .06	15.7 ± .08	15.9 ± .08

Inorganic Phosphorus.

	Group I	Group II	Group III
Control	4.14 ± .033	4.35 ± .05	4.11 ± .037
Light	4.14 ± .045	4.24 ± .05	3.84 ± .037
Dark	3.98 ± .037	4.10 ± .05	3.94 ± .034

Calcium-Phosphorus Ratio.

	Group I	Group II	Group III
Control	3.79 ± .04	3.61 ± .05	3.83 ± .04
Light	3.79 ± .05	3.62 ± .05	4.08 ± .04
Dark	3.97 ± .04	3.96 ± .05	4.09 ± .04

These figures show a very close agreement among animals of a given as well as of different groups. In Group I, there is a suggestion that the calcium content of the blood may have been higher in dark than in control or light animals, but in Groups II and III the difference is sufficient to be regarded as significant. In only one case, however, is there a significant difference between the values obtained for light and control animals (Group II). It is also of interest to note that while there is no definitely significant difference between the values for the 3 groups of control animals, there is a difference in the case of both the light and dark divisions of the 3 groups.

Comparison of the results for inorganic phosphorus and for the calcium-phosphorus ratio bring out a similar situation. In general, the value for inorganic phosphorus of animals in the dark is smaller than that for control or light animals, while the calcium-phosphorus ratio is larger. The only exception to this rule is in the results for inorganic phosphorus of light and dark animals in Group III and this may be a result of the change of environment, as suggested by the reversal of relations. Some of the effects indicated by the smooth curves are, therefore, borne out by a quantitative comparision of aggregate mean values. If a comparison is made of values obtained for successive phases of the reaction, differences are found between all classes of animals, and in some instances, they are even more clearly defined than those shown by aggregate mean values.

This does not prove, however, that the differences found are due to light effects. It is possible, but improbable, that by chance selection, the animals were distributed in such a way as to form groups with differences in the basic levels of calcium and inorganic phosphorus which were the same in all cases and that these differences were maintained with some variation throughout the period of observation. It is difficult to eliminate this possibility as there is no entirely satisfactory method of determining basic levels for the several groups of animals due to the disturbance of equilibrium following the change from outdoor to indoor life. This aspect of the problem may be clarified, however, by a comparison of results based on the relations shown by light and dark animals for different periods of the experiments. An examination of the figures given in Table I shows that up

to Nov. 19 the results for calcium on Group I were irregular with no clearly defined difference in levels. From Nov. 19 to Mar. 11, the general level of all values increased but not exactly to the same extent, while from Mar. 25 to May 18, there was a further increase among control and light animals but a decrease occurred in the dark division. In Group II, the differences for successive periods are not so clearly defined, but for comparative purposes the same divisions may be made. In the case of Group III, it will be necessary to adhere to the divisions made by experimental conditions. In this instance, nothing is known concerning basic levels of control, light, or dark animals. The first set of values corresponds with the values for the second period as given for Groups I and II, while the second and third groups of results represent effects produced by a change of environmental conditions. On this basis the mean values for calcium are as follows:

Group I.

	Oct. 8 to Nov. 12	Nov. 19 to Mar. 11	Mar. 25 to May 18
Control	14.91 ± .277	15.58 ± .080	16.04 ± .133
Light	14.59 ± .179	15.47 ± .067	15.79 ± .136
Dark	14.77 ± .106	15.83 ± .081	15.38 ± .134

Group II.

Control	14.42 ± .151	15.47 ± .091	15.87 ± .173
Light	14.09 ± .156	15.14 ± .080	15.20 ± .112
Dark	15.17 ± .235	15.87 ± .074	15.85 ± .124

Group III.

Control	15.5 ± .196	15.3 ± .084	16.1 ± .142
Light	15.4 ± .241	15.0 ± .069	16.1 ± .142
Dark	16.6 ± .226	15.4 ± .063	16.1 ± .134

On the same basis, the divisions that would be made in the results for inorganic phosphorus do not fall at exactly the same points and there is again a difference between Groups I and II. According to the

divisions indicated in Tables II and IV, the comparative results for inorganic phosphorus are as follows:

Group I.

	1	2	3
Control	4.42 ± .057	4.07 ± .052	3.83 ± .041
Light	4.51 ± .058	4.01 ± .063	3.64 ± .043
Dark	4.30 ± .036	4.15 ± .052	3.45 ± .044

Group II.

	1	2	3	4
Control	4.95 ± .120	4.83 ± .078	4.45 ± .072	3.75 ± .048
Light	4.58 ± .048	4.79 ± .083	4.04 ± .092	3.75 ± .041
Dark	4.80 ± .155	4.47 ± .070	4.04 ± .062	3.55 ± .051

Group III.

	1	2	3
Control	4.48 ± .083	4.05 ± .044	3.87 ± .056
Light	4.35 ± .085	3.74 ± .037	3.55 ± .056
Dark	3.91 ± .080	4.01 ± .044	3.82 ± .066

Ratio values might be compared in the same way but the results for calcium and phosphorus are sufficient to bring out the points of chief importance. In the first place, it is evident that the relative order of magnitude of the values obtained for calcium and phosphorus cannot be attributed to inherent differences in the basic levels of control, light, and dark animals. It is also evident that definite changes of level occurred in all classes of animals which involved changes in the relative magnitude of values from one period to another. This shows not only an appreciable degree of independence of action of factors responsible for these changes, but that the effect produced was influenced by the length of time animals were exposed to the action of a particular influence. The time influence is best illustrated by the results for dark animals of Group I which showed a clearly defined increase in calcium over a long period of time and a terminal decrease,

while for corresponding periods, inorganic phosphorus was maintained at a higher level than in control or light animals but eventually dropped to a much lower level.

With reference to the effects produced by a change of environment, as in Group III, it may be pointed out that the marked decrease in calcium with a slight, but apparently definite, increase in phosphorus shown by dark animals when placed in the light is conclusive evidence in itself that the first change of environment produced an effect. This result is supported, however, by the occurrence of a marked decrease in phosphorus in the light division. But, calcium did not increase as might have been expected or the increase came too late to affect the mean value for that period. This, in itself, is of importance as it suggests a difference in the rate of response or force of action of positive and negative influences following a prolonged exposure to an influence of the opposite kind.

From a quantitative analysis of the results, one may conclude that, in general, the conditions shown by the smoothed curves give a fair conception of the actual influence of environmental conditions on blood calcium and inorganic phosphorus. But, in attempting to define the action of this influence, it should be pointed out that there is evidence to show that light environment was not the only factor concerned in the production of the variations that occurred and, hence, that the influence exerted by light was subject to modification by other factors. Moreover, it appears that the effect produced in a given case depended to some extent upon the existing levels of calcium and phosphorus as well as the concentration of other substances in the blood which influenced the calcium and phosphorus content or equilibrium. Time is also an important factor as the effect produced varied with the length of exposure to a given influence.

The results obtained show conclusively that both absolute amounts of calcium and inorganic phosphorus in the blood of rabbits and the calcium-phosphorus equilibrium were affected by conditions of light environment which included no short wave-length ultra-violet. A distinction is to be made between effects produced by a change of environment and effects produced by prolonged exposure to a fixed environmental condition of a given nature.

With animals that had not been subjected to prolonged exposure

to a fixed environment, a change of environment produced a more or less marked disturbance of the calcium-phosphorus equilibrium extending over a period of 7 to 9 weeks. On the whole, the effect produced by a change from outdoor to indoor life was greater than that produced by a change from the diffuse light of the laboratory to neon light or darkness. The outstanding features of the effects produced by a change from outdoor to indoor life were first, an increase, and then a decrease in the calcium content of the blood, with changes in inorganic phosphorus in the opposite direction, so that the ratio of calcium to phosphorus was greatly increased and then diminished. These effects were modified by exposure to neon light and by exclusion of light. A change of environment after prolonged exposure to a fixed environment produced only a slight and transient disturbance of equilibrium with a marked delay in the development of effects characteristic of the new environment.

The initial effects of exposure to a fixed environment of neon light or darkness are uncertain. The first definite effect of the exclusion of light on the calcium content of the blood was an increase with the maintenance of a level in the upper ranges of normal; the effect of neon light was a decrease with the maintenance of a level in the lower ranges of normal. Both conditions produced a decrease of inorganic phosphorus, but the decrease was less among animals exposed to neon light and there was evidence of a tendency to maintain a higher level or to prevent a decrease to an extremely low level. The effect of neon light was maintained throughout the period of observation, subject to the modifying influence of other factors, but the early effect of exclusion of light lasted for only a few months; the terminal effect was a decrease of both calcium and inorganic phosphorus.

With the increase in calcium and the decrease in inorganic phosphorus there was a marked increase in the calcium-phosphorus ratio of animals in the dark and this increase continued until near the end of the experiments despite the occurrence of a decrease in calcium. The calcium-phosphorus ratio of animals exposed to neon light increased less and here again there was an obvious tendency to maintain a more stable equilibrium and to prevent the development of an excessively high ratio of calcium to inorganic phosphorus.

The results obtained for animals living in the diffuse light of the

laboratory differed from those of animals living under fixed environmental conditions in several significant respects. They showed wider variations within a given period of time and more frequent fluctuations; calcium tended to be higher than for animals exposed to neon light but lower than for animals in the dark, while inorganic phosphorus was, for the most part, higher than in either of the other groups, but at times was definitely lower. The calcium-phosphorus ratio was very irregular, at times it was much higher than that of other animals, and at other times much lower, due to the occurrence of wide variations from a general level which was somewhat lower than that of animals exposed to neon light. The outstanding features of the condition presented by these animals, as compared with animals living under fixed environmental conditions, was, therefore, the instability of the calcium-phosphorus equilibrium.

While the results have not been analyzed in detail from the point of view of color or breed, sufficient evidence has been presented to show that the reaction to environmental conditions or susceptibility to the light influence is not the same for all classes of animals, but it is uncertain whether this difference is attributable to color or to some more fundamental difference in the constitution of the animal organism.

The data have also been analyzed from the standpoint of the distribution of individual values, but since this form of analysis reveals essentially the same differences as have been noted above, the results are omitted.

The features of the results obtained in these experiments that are of especial interest from the point of view of their bearing on the reaction of the animal organism to environmental conditions are the profound effects produced by a change of light environment and the relation of the effects produced by exposure to light and by exclusion of light to conditions that occur normally as a function of age. The depression or suspension of growth activity and the occurrence of certain changes in organic constitution, associated with an increase in blood calcium and a decrease in inorganic phosphorus, with a marked increase in the calcium-phosphorus ratio produced by deprivation of light are conditions analogous to the changes that occur with advancing age. The effects produced by neon light, on the other hand, suggest that its

action tended to prevent the occurrence of premature aging or to maintain conditions that are compatible with vigorous functional activity. Moreover, available evidence seems to indicate that this analogy between the action of light and changes that occur with age is equally applicable to the effects produced by light environment on susceptibility and the reaction to disease.

In conclusion, it may be said that the results of this series of experiments, including those on nutrition and growth and on organic constitution, show that, under appropriate conditions, it is highly probable that light of any kind is capable of producing a biological effect even though the energy involved may be extremely small.

SUMMARY.

A series of experiments was carried out for the purpose of determining whether a light environment comprising radiations of comparatively long wave-length and only a small amount of energy was capable of affecting the chemical equilibrium of the blood as indicated by the calcium and inorganic phosphorus content of the blood of normal rabbits. A study was made of effects produced by prolonged exposure to fixed environmental conditions (neon light and darkness) as compared with a varying environment of diffuse, filtered sunlight and by a change from one environment to another.

It was found that the chemical equilibrium of the blood was definitely affected by the conditions employed and that the effects produced could be correlated with differences in organic constitution on the one hand, and on the other, with certain differences in the functional activity of the same animals, involving nutrition and growth and the proliferative activity of hair follicles.

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STUDIES ON THE SENSITIZING PROPERTIES OF THE BACTERIOPHAGE.

By CLAUS W. JUNGEBLUT, M.D., and EDWIN W. SCHULTZ, M.D.

(*From the Department of Bacteriology and Experimental Pathology, Stanford University, California.*)

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INTRODUCTION.

It is surprising that among the numerous investigations on the properties of the bacteriophage no systematic inquiry has been made to determine whether the bacteriophage is capable of sensitizing the tissues of experimental animals, a property which we commonly associate with the antibody-producing function of more complex antigenic substances. The few suggestive observations made do not serve to cast any definite light on this problem. Thus, d'Herelle (1) states, in a brief note, that repeated injections of rabbits and mice with Shiga bacteriophage may sensitize these animals to the Shiga toxin, a condition for which he has coined the term "antiphylaxis." He claims that a form of hypersensitiveness, passive in character, may also be obtained by injecting animals with mixtures of sublethal doses of Shiga toxin and specific antiphage serum. Otto and Munter (2) have not been able to observe the regular occurrence of this phenomenon. Similar experiments by Gratia (3) and by Hauduroy (4) with staphylococcus bacteriophage seem to indicate that prolonged immunization with this phage may not only cause emaciation and premature death of some animals, but that the surviving animals show upon reinoculation an increased susceptibility to the same organisms. Although we have occasionally observed slowly progressing emaciation of rabbits during immunization with staphylococcus bacteriophage, we are inclined to question whether one is justified in interpreting any of the above phenomena as manifestations of true or typical anaphylaxis.

In view of the general trend of more recent studies on the antigenic properties of the bacteriophage (Schultz (5)) which suggest that the

antigenic action of the bacteriophage is strictly limited to a capacity of inducing the formation of specific neutralizing antibodies, we felt that it would be of fundamental importance to determine whether or not the bacteriophage is capable of producing a sensitization effect in suitable animals. Some of the contradictory and confusing results obtained in the past with the complement fixation test in studies on the antigenic properties of the bacteriophage and the ultraviruses, generally, appear to be clearly traceable to unspecific factors, not always fully recognized in the evaluation of the results. The anaphylactic reaction, on the other hand, is characterized not only by an extraordinary degree of specificity but also sensitivity, so that results secured with this procedure would seem to carry definitely more weight than serological reactions carried out *in vitro*.

EXPERIMENTAL WORK.

Preliminary attempts to elicit in guinea pigs, actively sensitized to various bacteriophages, typical symptoms of anaphylactic nature by intravenous reinjection of the same antigen, had to be abandoned, because it appeared definitely impossible to produce either fatal shock or sufficiently pronounced symptoms by this method. We, therefore, decided to make use of the more delicate reactivity of the isolated uterine muscle as it may be measured by the Schultz-Dale method. The procedure of passive anaphylaxis which has proved so successful in recent studies of Tomcsik and Kurotchkin (6) in determining the sensitizing properties of bacterial carbohydrates, was given a trial. The interval between intraperitoneal injection of the antiserum and the testing of the uterine strip varied in these few preliminary experiments from 24 hours to 5 days. The guinea pigs, however, failed to become sufficiently sensitized, although they had been prepared with large doses of potent antiphage rabbit sera. We have, therefore, primarily confined ourselves to a study of active anaphylaxis, using as test objects the isolated uterine horns of *actively* sensitized young, virgin guinea pigs.

A number of modifications of the original Schultz-Dale technic have been described. We have adopted an outfit recommended by Hanzlik and De Eds (7) which in our opinion greatly facilitates the study of surviving organs. As an

immersion fluid in the bath we used freshly prepared Tyrode solution. Other technical details of the actual test do not require any further description since in every other respect we have followed the customary procedures. In the following lines we have given a description of the methods employed in the preparation of the various reagents and of the procedure of sensitizing the animals.

Preparation of Reagents.—Since many of the positive results reported with the complement fixation and precipitin tests on antiphage sera, credited to the bacteriophage itself, are undoubtedly traceable to bacterial residues in the filtrates, we endeavored in our experiments to eliminate the rôle of these concomitant antigens by employing a bacteriophage capable of lysing two distinct bacterial species. By sensitizing, for example, with phage-lysed Flexner bacilli and testing for anaphylaxis with colon bacilli lysed by the same bacteriophage, it became clear that any reaction which might be elicited could only be attributable to a direct antigenic effect of the bacteriophage itself. In order to further obviate any possible interference by extraneous antigenic substances contained in the culture substrate, we have attempted to grow and lyse our organisms in a synthetic medium devoid of protein. While we succeeded in obtaining good growth and also complete bacteriophage lysis of the colon bacillus, cultivated in Uschinsky's medium, it proved impossible for us to adapt the Flexner bacilli to this artificial medium. We have therefore, in the case of Flexner antigens, varied the medium for sensitization and the anaphylactic test by using Martin's broth in preparing the sensitizing lysates and beef infusion broth for the preparation of the lysate used in the bath. As a further check, a small number of guinea pigs were sensitized against Flexner lysates, which had been trypsinized before use, a method that may be employed to free bacteriophage suspensions from residual bacterial and other proteins (Arnold and Weiss (8)) without injuring the bacteriophage. We have moreover on general principles reduced the presence of unlysed bacterial proteins in our lysates to a minimum by employing a bacteriophage of maximum virulence for both the Flexner and colon bacilli in question. In addition, the lysed cultures were always filtered, shortly after complete lysis had been attained, through a Chamberland L 3 candle, well before the onset of any possible secondary growth of resistant bacterial cells.

Method of Sensitization.—Experiments of Zinsser and his associates (9, 10) have shown with sufficient clarity that, in order to obtain positive results with any degree of regularity in the difficult and much contested field of bacterial anaphylaxis, it is necessary to subject the animals undergoing sensitization to an intensive course of treatment. Our guinea pigs therefore were all given a series of seven subcutaneous injections of the respective antigens administered at 3 day intervals, the doses ranging from 0.5 cc. to 3 cc. Some animals were lost during the course of sensitization. The interval between the last sensitizing injection and the sacrificing of the animal for the test varied between 3 and 5 weeks.

We have run two large series, comprising a total of 62 sensitized animals. In addition, a total of twelve normal guinea pigs was included in order to accurately determine the maximum dose of the different lysates and broth cultures, which

failed to cause irritation of the normal uterine horn. This dose varied with the different preparations from 4 to 8 cc., added to a bath of 50 cc. capacity. An amount well below (one-half to one-fourth of the minimum irritating dose) was used in testing the sensitized uterine strips. It should be mentioned in this connection that it was not possible to add even traces of trypsinized material to the bath without provoking maximum contraction of the normal muscle. On the other hand, the Uschinsky medium caused an immediate marked depression in the tonus of the normal muscle. We have, therefore, refrained from employing either one of the media as a vehicle for any reagents used in the final test.

The complete protocols of the two experimental series are as follows:

Series I.—One group of six guinea pigs was sensitized against phage-lysed colon bacilli, grown in Uschinsky's medium (Phage 1,[1] K-13), another group of six animals against the intact, phage-free colon bacilli (K-13), naturally the identical bacterial strain. Six guinea pigs received sensitizing injections of phage-lysed Flexner bacilli, grown in Martin's broth (Phage 1, I-13), and an equal number was sensitized in the same manner against the intact, phage-free Flexner culture (I-13). Four guinea pigs were sensitized with the Flexner lysate, subjected to thorough trypsinization[2] (Phage 1, I-13 trypsinized), while four more animals were injected with uninoculated Martin's broth and Uschinsky's medium, respectively, for purpose of control.

In testing our guinea pigs for acquired hypersensitiveness, we have experimented with various combinations of sensitized uterine horns and test reagents. As previously outlined, animals sensitized to the two phage lysates (Flexner or colon) were tested not only with the homologous bacteriophage suspensions, but also with the same bacteriophage, but propagated on the heterologous organisms. These same animals were also tested for anaphylaxis with autolysates of the corresponding bacteria, obtained by allowing broth cultures in sealed tubes to remain in the incubator for 3 to 4 weeks. The term autolysate as used here and later in the text is meant to designate phage-free broth cultures, partially cleared by spontaneous bacterial autolysis, and bearing no relationship to the bacteriophage phenomenon. As regards animals sensitized to the intact bacterial cells (Flexner or colon), they were subjected to a test not only with the corresponding bacterial autolysates but also with the homologous phage lysates.

Although not all of the uterine strips reacted in a reliable and clean-cut manner, some failing to respond entirely, we secured uniform and significant tracings with the majority of animals tested. Our

[1] Phage 1 is an antidysentery phage obtained from Dr. d'Herelle and isolated originally by him from the feces of a goose.

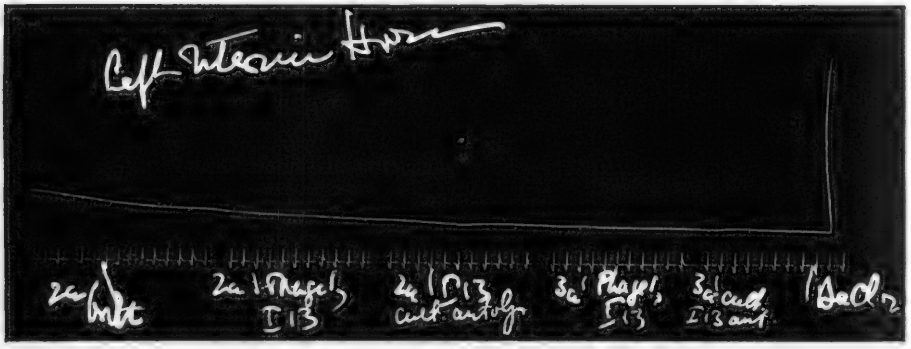
[2] It is common knowledge to students of bacteriophagy that bacteriophages, with certain exceptions (Schultz (11)), resist the action of tryspin.

observations in this series demonstrated very clearly three outstanding phenomena, which may briefly be summarized as follows: First, we obtained with great regularity marked contractions of the uterine strips of guinea pigs, sensitized to phage-lysed bacterial cultures, upon adding to the bath homologous *phage lysates*, while no reaction followed the addition of homologous bacterial *autolysates* to such muscles. Secondly, marked contractions of the uterine horns, taken from animals sensitized to the normal intact bacterial cells, occurred following the addition of the homologous bacterial *autolysates* to the bath, but no such reactions were demonstrable upon adding the corresponding *phage lysates*. These phenomena were equally well marked in both groups, guinea pigs sensitized to the Flexner antigens and those sensitized to the colon antigens. The specificity of these positive reactions is further indicated by the complete and specific desensitization of the sensitized muscle, which in most instances followed immediately after the initial contraction. The third fact, which these experiments brought to light, was the absence of any contraction of phage-sensitized strips, when these were tested against the heterologous phage lysates, *i.e.*, guinea pigs sensitized to Flexner lysates showed no reaction whatsoever with colon lysates, and *vice versa*, Flexner lysates were entirely inactive on strips sensitive to colon lysates, although the bacteriophage in each instance was the same. The control animals, sensitized to the uninoculated Martin and Uschinsky broth, had acquired no demonstrable hypersensitiveness to the respective media. Likewise, strips from animals sensitized to trypsinized Flexner lysates, did not react upon contact with the non-trypsinized Flexner lysates.

It appears from these experiments that phage-lysed bacteria exhibit antigenic properties quite distinct and different from those belonging to bacteria which have been allowed to undergo disintegration by spontaneous autolysis. In addition, it would seem that the sensitizing effect of a bacteriophage suspension is due exclusively to the dissolved bacterial protein, *altered by phage action*, and not to the bacteriophage itself, because cross-tests, in which the bacteriophage represented the only common antigen, gave consistently negative reactions. It is of interest and seemingly significant that animals, sensitized to trypsinized Flexner lysates, failed to respond when tested

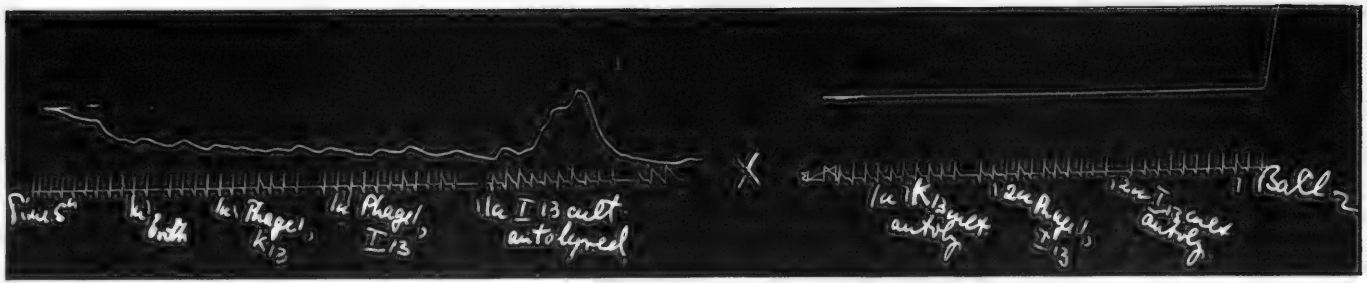
GRAPH 1. Uterine strip from normal guinea pig (right horn).

(*a*) 2 cc. I-13 culture (Flexner autolyzed).....no reaction.
(*b*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli) " "
(*c*) 2 " K-13 culture (*coli* autolyzed)..... " "
(*d*) 2 " Phage 1, K-13 (phage-lysed *coli* bacilli)... " "
(*e*) $BaCl_2$.....maximum contraction.



GRAPH 2. Uterine strip from normal guinea pig (left horn).

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli)..... " "
(*c*) 2 " I-13 culture (Flexner autolyzed)..... " "
(*d*) 3 " Phage 1, I-13 (phage-lysed Flexner)..... " "
(*e*) 3 " I-13 culture (Flexner autolyzed)..... " "
(*f*) $BaCl_2$.....maximum contraction.



GRAPH 3. Uterine strip from Guinea Pig 16. Sensitized to I-13 culture (Flexner bacilli) (left horn). June 8, 1928.

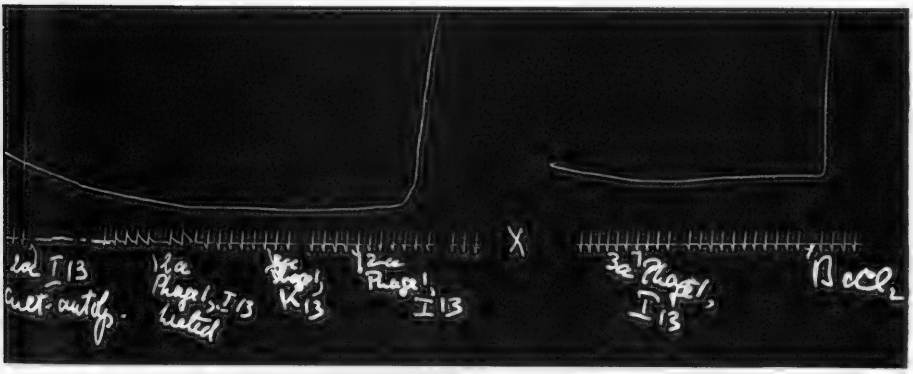
(*a*) 1 cc. broth.....no reaction.
(*b*) 1 " Phage 1, K-13 (phage-lysed *coli* bacilli).. " "
(*c*) 1 " Phage 1, I-13 (phage-lysed Flexner bacilli)..... " "
(*d*) 1 " I-13 culture (Flexner, autolyzed).....contraction.
(*e*) Bath changed.
(*f*) 1 cc. K-13 culture (*coli*, autolyzed).....no reaction.
(*g*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli)..... " "
(*h*) 2 " I-13 culture (Flexner, autolyzed)..... " " *Desensitization.*
(*i*) $BaCl_2$.....maximum contraction.

GRAPH 4. Uterine strip from Guinea Pig 8. Sensitized to Phage 1, I-13. Phage-lysed Flexner bacilli (right horn). May 7, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, K-13 (phage-lysed colon bacilli). " "
(*c*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli).....contraction.

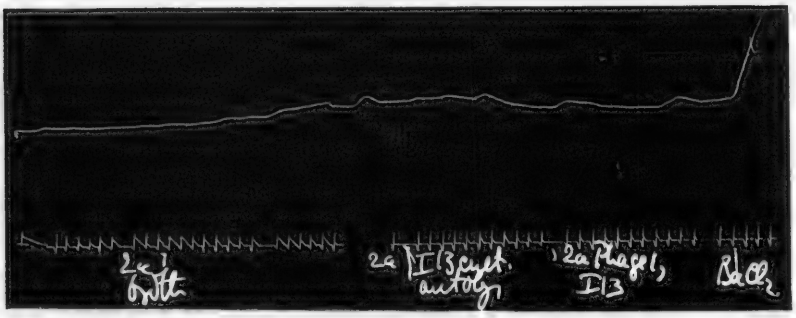
GRAPH 5. Uterine strip from Guinea Pig 8. Sensitized to Phage 1, I-13. Phage-lysed Flexner bacilli (left horn). May 7, 1928.

(*a*) 2 cc. Phage 1, K-13 (phage-lysed colon bacilli).....no reaction.
(*b*) 2 " I-13 culture (Flexner)..... " "
(*c*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli).....contraction.



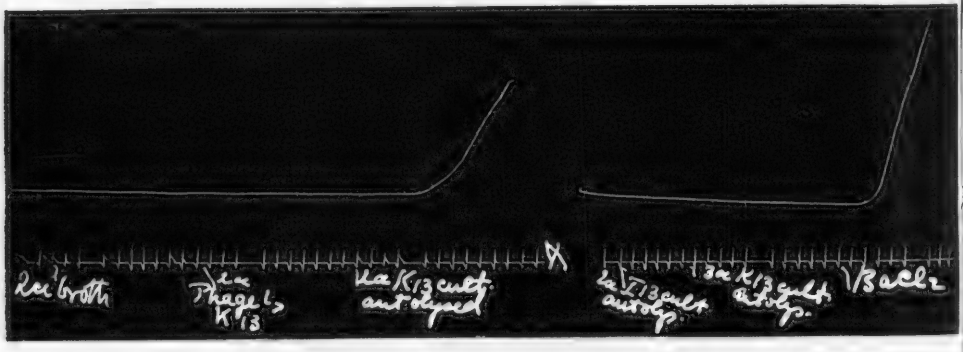
GRAPH. 6. Uterine strip from Guinea Pig 26. Sensitized to Phage 1, I-13. Phage-lysed Flexner bacilli (left horn). July 25, 1928.

(*a*) 2 cc. I-13 culture (Flexner, autolyzed).....no reaction.
(*b*) 2 " Phage 1, I-13 (*heated* phage-lysed Flexner bacilli)..... " "
(*c*) 3 " Phage 1, K-13 (phage-lysed colon bacilli). " "
(*d*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli) not heated.....contraction.
(*e*) Bath changed.
(*f*) 3 cc. Phage 1, I-13 (phage-lysed Flexner bacilli).....no reaction. *Desensitization.*
(*g*) $BaCl_2$.....maximum contraction.



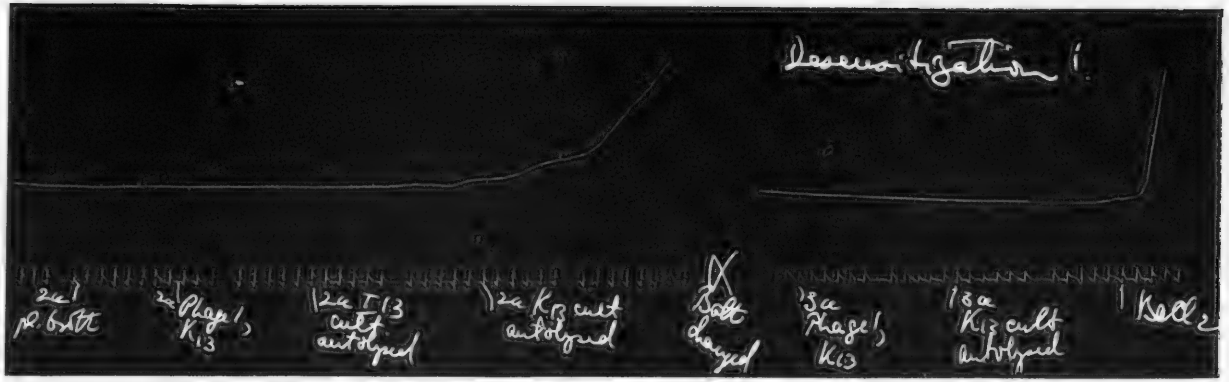
GRAPH 7. Uterine strip from Guinea Pig 34. Sensitized to Phage 1, I-13, trypsinized. Phage-lysed Flexner bacilli, trypsinized (right horn). July 25, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " I-13 culture (Flexner, autolyzed)..... " "
(*c*) 2 " Phage 1, I-13 (phage-lysed Flexner bacilli)..... " "
(*d*) $BaCl_2$.....maximum contraction.



GRAPH 8. Uterine strip from Guinea Pig 29. Sensitized to K-13 culture (colon bacilli) (left horn). August 2, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, K-13 (phage-lysed colon bacilli).. " "
(*c*) 2 " K-13 culture (colon autolyzed).....contraction.
(*d*) Bath changed.
(*e*) 2 cc. I-13 culture (Flexner autolyzed).....no reaction. *Desensitization.*
(*f*) 3 " K-13 culture (colon, autolyzed)..... " "
(*g*) $BaCl_2$.....maximum contraction.

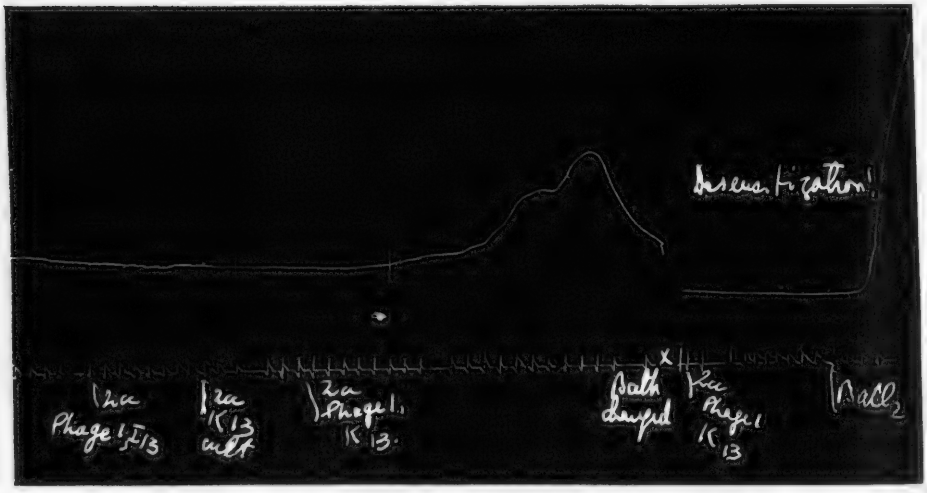


GRAPH 9. Uterine strip from Guinea Pig 30. Sensitized to K-13 culture (colon bacilli) (right horn). August 6, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, K-13 (phage-lysed colon bacilli). " "
(*c*) 2 " I-13 (Flexner) culture, autolyzed..... " "
(*d*) 2 " K-13 (colon) culture, autolyzed.....contraction.
(*e*) Bath changed.
(*f*) 3 cc. Phage 1, K-13 (phage-lysed colon bacilli). no reaction.
(*g*) 3 " K-13 (colon) culture, autolyzed..... " " *Desensitization*.
(*h*) $BaCl_2$.....maximum contraction.

GRAPH 10. Uterine strip from Guinea Pig 10. Sensitized to Phage 1, K-13. Phage-lysed colon bacilli (right horn). May 15, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " K-13 (colon) culture..... " "
(*c*) 2 " Phage 1, K-13 (phage-lysed colon bacilli)..contraction.



GRAPH 11. Uterine strip from Guinea Pig 11. Sensitized to Phage 1, K-13. Uschinsky medium. Phage-lysed colon bacilli (right horn). May 15, 1928.

(*a*) 2 cc. Phage 1, I-13 (phage-lysed Flexner bacilli).....no reaction.
(*b*) 2 " K-13 culture (colon)..... " "
(*c*) 2 " Phage 1, K-13 (phage-lysed colon bacilli)..contraction.
(*d*) Bath changed.
(*e*) 2 cc. Phage 1, K-13 (phage-lysed colon bacilli).....no reaction. *Desensitization.*
(*f*) $BaCl_2$.....maximum contraction.

GRAPH 12. Uterine strip from Guinea Pig 31. Sensitized to Phage 1, K-13, trypsinized. Phage-lysed colon bacilli, trypsinized (left horn). August 7, 1928.

(*a*) 2 cc. broth.....no reaction.
(*b*) 2 " Phage 1, K-13 (phage-lysed colon bacilli).....practically no reaction.
(*c*) 2 " K-13 (colon) culture, autolyzed.....no reaction.
(*d*) Bath changed.
(*e*) 2 cc. Phage 1, I-13 (phage-lysed Flexner bacilli).....no reaction.
(*f*) 2 " Phage 1, K-13 (phage-lysed colon bacilli). " "
(*g*) 2 " K-13 (colon) culture, autolyzed..... " "
(*h*) $BaCl_2$.....maximum contraction.

with the homologous non-trypsinized lysate. This would indicate that the new antigenic complex of phage-lysed bacilli had been destroyed by the action of trypsin in the same manner as the normal bacterial proteins are digested by that ferment.

Series II.—In the attempt to verify the results obtained with the first series of experiments we have run another series under similar experimental conditions, involving virtually the same number of sensitized guinea pigs, the only exception being that in this series the groups of animals, sensitized to the normal Flexner or colon cultures, were injected with spontaneously autolyzed cultures instead of with intact bacterial cells. It was thought desirable to include this modification, in order to approach as closely as possible the physical state and high dispersion of bacterial proteins presumably provided by phage lysis.

The results obtained in this second series were in principle exactly the same as those observed in the former series. We shall therefore omit a detailed description and instead refer the reader to Graphs 1 to 12 which record individual experiments of both series. These graphs also give full particulars as to dosage, time interval, intensity of contraction, desensitization and control measures; they have been selected from a large number of records and are representative of the types of reactions obtained with the various combinations in question.

DISCUSSION.

The observations made in this paper are of interest because they demonstrate two fundamental facts: First, it *would seem that the bacteriophage virus*[3] *per se is not capable of sensitizing guinea pigs to a degree and extent detectable by ordinary experimental methods.* This conclusion is made practically inevitable by the negative outcome of cross-test, in which the bacteriophage constituted the only common antigen. Secondly, it *appears that the bacterial protein, after lysis of the organisms by the bacteriophage, acquires new antigenic properties which differ radically from those of the intact bacteria or bacterial cells dissolved by spontaneous autolysis.*

As to the first conclusion, our results agree with the trend of recent studies on the antigenic properties of the bacteriophage which suggest that antiphage sera, produced with phage antigens, freed from bac-

[3] The bacteriophage is designated a virus here, quite without reference to the disputed question as to its living or non-living nature.

terial proteins by trypsinization, contain only specific neutralizing antibodies and do not possess either complement-fixing or precipitating qualities (Arnold and Weiss). The inability of the bacteriophage in itself to produce either a passive or active sensitization effect would further serve to emphasize the peculiarly restricted antigenic action of this virus, a fact which has been brought out more fully in the recent studies by Schultz, Quigley and Bullock (12) on the antigenic properties of the bacteriophage. The bacteriophage in this respect resembles the ultraviruses and toxins, which, as has been pointed out by Schultz in a recent critical review (5), seem to fall into a special category of antigenic substances which apparently do not engender specific complement-fixing or precipitating antibodies during the immunization of experimental animals.

The fact that phage lysis of bacteria gives rise to new and immunologically distinct antigenic complexes is of fundamental importance, inasmuch as it suggests that the lysis brought about by phage action is not merely a physical phenomenon, but appears to be intimately associated with a profound biochemical change of the bacterial protein. This conclusion seems the most logical interpretation of our results, although it may be possible that some other mechanism, at present not clear to us, underlies the observed phenomena. It is of interest that recent researches on certain filtrable viruses of plants also indicate an alteration of the protoplasma of the host by the pathogenic activities of the implanted viruses. Thus Dvorak (13), basing her conclusions on the precipitin test, has reported a new specificity for plant proteins diseased with the virus of potato mosaic. Purdy's (14) observations on tobacco mosaic virus are of a similar nature. All these contributions strongly suggest that the propagation of ultramicroscopic agents of disease, because of their more intimate contact with the internal mechanism of the cell and because of the peculiar morbid changes which they induce, leads to a more or less complete transformation or to a denaturization of the protoplasm of the cells of the affected host.

It is difficult, *a priori*, to reconcile our observations on the alteration of bacterial protein by phage lysis with the findings of numerous authors secured with the complement fixation, precipitin and agglutination tests, all of which would seem to indicate that antiphage sera

contain a definite quota of specific antibodies which can be shown to react in various ways with the unchanged bacterial antigen. This objection takes for granted that rabbits behave like guinea pigs during immunization with the bacteriophage and, furthermore, that sensitization, as measured by the contraction of the isolated muscle, registers an immunity response identical with the one indicated by the serological reactions *in vitro*. Without discussing for the present the validity of such arguments, there are other factors which would seem to go far towards explaining the discrepancy on a satisfactory basis. It is by no means easy to secure under all conditions a phage lysate, in which all available bacterial protein has been completely transformed into the new antigenic complex by the action of the bacteriophage, since the virulence of many bacteriophages is so limited that, in spite of macroscopic clearing of the culture, an appreciable number of bacterial cells must escape the primary lysis. It has to be considered furthermore that even with bacteriophages of maximum virulence, there is frequently an occurrence of secondary growth of resistant bacterial cells after the acme of lysis is over. These two factors, which depend very much on the composition of the medium, the temperature of incubation and the interval between filtration of the lysate and the completion of lysis, undoubtedly explain the variation in composition and, consequently, in antigenic behavior of different bacteriophage suspensions. We believe that our results with the sensitized muscle have been of such a uniform character and have shown practically no overlapping between the antigenic action of autolysates and phage lysates, because special precautions were taken to obtain as ideal bacteriophage preparations as possible by employing only a lytic principle of maximum virulence for the susceptible organisms and by filtering the lysed cultures immediately after the acme of lysis.

As regards our reactions with strictly bacterial anaphylaxis, we happened to have experienced no difficulty in obtaining maximum contractions of uterine strips, sensitized to normal bacteria, by testing them with autolyzed or even intact homologous bacteria, while Zinsser and his associates stress the necessity of using bacterial extracts, prepared in a laborious way for the anaphylactic test, in order to obtain enough of the reacting proteins in solution.

SUMMARY AND CONCLUSIONS.

1. Marked specific contractions of the uterine horns of guinea pigs, actively sensitized, to phage-lysed Flexner bacilli or to colon bacilli, lysed by the same bacteriophage, occurred on testing either series for anaphylaxis with the homologous phage lysates. These reactions, however, were not due to an antigenic function of the bacteriophage itself, because no reaction whatsoever occurred when the same bacteriophage, propagated on the heterologous organisms, was substituted in the anaphylactic test.

2. Specific uterine reactions of marked intensity were obtained in guinea pigs, actively sensitized to intact or autolyzed Flexner or colon bacilli, respectively, by testing either series for anaphylaxis with homologous, phage-free bacterial antigens.

3. No reaction occurred by testing the uterine strips of animals, sensitized to *intact or autolyzed* bacilli (either Flexner or coli), for anaphylaxis with homologous *phage lysates* and, *vice versa*, there was no contraction of uterine strips sensitized to *phage lysates* upon contact with homologous bacterial *autolysates*.

4. The observations made in this paper suggest that a new and immunologically distinct antigenic complex arises from the bacterial protein after lysis of the organisms by the bacteriophage.

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STUDIES OF CALCIUM AND PHOSPHORUS METABOLISM.

V. A Study of the Bone Trabeculæ as a Readily Available Reserve Supply of Calcium.*†

By WALTER BAUER, M.D.,‡ JOSEPH C. AUB, M.D., and FULLER ALBRIGHT, M.D.§

(*From the Medical Clinic of the Massachusetts General Hospital, Boston.*)

Plates 13 to 17.

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By far the largest part of the calcium in the body takes part in making up the skeletal structure. Metabolism studies have shown that there are many factors which add or subtract from the body's supply of calcium. Thus pregnancy, lactation, a low calcium intake, ingestion of acids, administration of parathormone[1] and many other influences serve to increase the demand for calcium from the body. The question arises: Is there a special storehouse of calcium, or is the whole body skeleton at the mercy of these various factors? A very significant observation in this connection was made by Hunter and Aub (1). In patients with chronic lead poisoning they observed an increased lead excretion, which accompanied the increased calcium excretion following the administration of parathormone. If these same patients were then put on a high calcium diet so as to store up the amount of calcium lost during the parathormone medication, and were then given a second course of parathormone, they failed to show an increased lead excretion although the calcium excretion was

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† Read before The American Society for Clinical Investigation, May 2, 1927.

‡ Medical Resident, Massachusetts General Hospital.

§ Research Fellow, Massachusetts General Hospital and Harvard Medical School.

[1] Parathormone is the trade name for Collip's parathyroid extract.

elevated as markedly as before.[2] This observation suggests that the first parathormone medication withdrew calcium and the lead deposited with it from some storehouse of reserve calcium; that this store was replenished during the period of high calcium intake; and that finally the second parathormone medication affected only the newly deposited store, and did not affect the skeleton at large, which, of course, still contained considerable lead. It was further noted that following a course of treatment with parathormone, the small daily excretion of lead continued as before. This suggests a constant excretion from some source, such as the cortex of bone, and that this represents the normal catabolism of the skeletal structure.

While working on calcium metabolism in animals, we had noted that the amount of cancellous bone varied considerably in different animals but no note was made whether these variations were associated with any form of therapy or diet. This raised the question: Are the trabeculæ a reserve supply of calcium? Is it possible that the calcium in the bones can be divided into two parts;—a larger portion making up the structural part of the bone, which functions as a mechanism for body support, and a smaller, less stable portion acting as a reserve deposit for use when the body needs calcium or inorganic salts? The anatomical situation of the trabeculæ is in accord with such a theory. They are most numerous at the epiphyseal ends of the bones, where the blood supply is greatest. These delicate, lace-like structures, in close relation to the bone marrow, are well situated to receive calcium deposits in times of calcium excess, and to give up calcium in times of calcium demand. The shafts of the bones are not so well situated for this function.

EXPERIMENTS.

In an attempt to see whether the bone trabeculæ act as a reserve supply of calcium the following animal experiments were planned.

Experiment 1. Effect of Parathormone Administration on the Trabeculæ of Rabbits.

Parathormone administration is one of the most effective ways of increasing the calcium excretion and thus producing a negative

[2] See Hunter, D., and Aub, J. C., *Quart. J. Med.*, 1927, xx, 123, Chart 1.

calcium balance (1, 2). Therefore, we wished to determine its effect on the trabeculæ.

Experiment.—A litter of eight young rabbits was used. At the onset of the experiment they were 5 weeks old. Four were used as controls and four for parathormone injections. They were kept in the same bin, on a normal diet, high in calcium content. Early in the experiment Rabbits 334 (extract) and 274 and 267 (controls) died. The others were kept for a period of 91 days. The animals were given the parathyroid extract in gradually increasing doses until they were all receiving 8 units per day. (See Table I.) This dose they received for from 56 to 74 days of the period.

At the end of the 91 day period (24 hours after the last injection) all animals were anesthetized with ether and blood samples drawn. They were then killed, their blood serum analyzed for calcium (Table I) and their bone structures studied.

TABLE I.

Rabbit	Blood serum calcium	Total No. of units of parathormone injected
	mg. per 100 cc.	
301*	15.5	514
298*	14.2	514
327*	14.3	628
297†	11.6	
314†	10.3	

* Rabbits receiving parathormone.

† Control litter mates.

It is well shown in the x-ray plates (Fig. 1) that the bones of the extract animals (Nos. 298, 301, 327) contain much less calcium than do the bones of their control litter mates (Nos. 297 and 314). This is most marked in the trabeculæ at the epiphyseal end, particularly of the metacarpal bones. Fig. 2 shows the cross-section of the femora from each animal. This photograph clearly demonstrates that there are fewer trabeculæ in the extract animals than in the controls.

Conclusion to Experiment 1.—The trabeculæ are diminished following long continued parathormone administration in rabbits. No gross effect was noted on the cortex of the bone.

Experiment 2. Effect of High and Low Calcium Diets on the Trabeculæ of Adult Cats.

A diet deficient in calcium results in a negative calcium balance (3–5). On a low calcium diet as used in our experiments, we have found

that an average sized cat has an average negative calcium balance of approximately .3 gm. per week. In cats starving for a month, Sedlmair (6) was able to demonstrate a calcium loss of 1 per cent of the total body weight, or 2.5 per cent to 11.6 per cent calcium oxide loss from the bones. Wellmann (7) confirmed these observations working with rabbits. The opposite of this (a high calcium diet) results in a positive calcium balance (5, 8, 9). Where does this calcium loss come from and where is the excess deposited?

TABLE II.

Cat	Sex	Type of diet	Days on diet	Total Ca intake for exp.	Daily Ca intake
				gm.	*gm.*
337	♀	Low Ca diet	84	1.29	.016
337		High Ca diet	274	144.70	.528
338	♀	Low Ca diet	84	1.19	.014
338		High Ca diet	156	38.70	.248
339	♀	Low Ca diet	84	1.28	.015
339		High Ca diet	160	68.80	.430
341	♂	Low Ca diet	84	1.19	.014
341		High Ca diet	267	68.20	.257
342	♂	High Ca diet	79	22.60	.286
342		Low Ca diet	269	3.84	.014
343	♂	High Ca diet	79	48.20	.610
343		Low Ca diet	267	3.47	.013
344	♂	High Ca diet	80	31.15	.389
344		Low Ca diet	379	4.66	.012
345	♂	High Ca diet	80	30.06	.382
345		Low Ca diet	161	2.11	.013
346	♀	High Ca diet	79	29.10	.350
346		Low Ca diet	157	1.83	.012

The separate protocols for these animals are appended.

Experiment.—Five cats were placed on a high calcium[3] diet and four on a low calcium diet.[4] At the end of several months the left fore leg was amputated at the shoulder joint under ether anesthesia, then the diets were reversed. After a similar period of time the animals were sacrificed, thus allowing comparison of the humerus representing the high calcium diet period, with the humerus of the low calcium diet period.

[3] High calcium diet consists of milk, and in one animal milk and raw eggs.

[4] Low calcium diet consists of cooked meat, fresh, raw liver and water. Cats stay in excellent condition on this diet even if it is given them for many months.

The bones were all prepared in the same manner, the same length of time being taken for each process. They were cleaned, scraped and cross-sectioned. The marrow contents were removed by burning in an electric furnace. Thus each animal served as his own control and such factors as age, sex, activity and season do not enter into the results obtained. Table II gives in summary form the average daily calcium intake, the total calcium intake for each period and the length of time the animal was kept on each diet.

Figs. 3 to 6 show very well the results obtained from this experiment. Regardless of whether an animal is first placed on a high or low calcium diet, the result is always the same. The bone removed after the period of high calcium feeding contains many trabeculæ and its opposite member representing the period of low calcium diet shows a marked reduction in the number of trabeculæ. From Figs. 5 and 6, it will be seen that these changes are most marked in Cats 343, 337, 345, 342, 339, 346 and 344. These animals had the largest daily calcium intake during the period of high calcium feeding (see Table II). The changes are least marked in Cats 338 and 341 who had the lowest daily calcium intake when on a high calcium diet. The absence of trabeculæ is most marked in Cat 344 (see Fig. 6). It was on a high calcium diet first (80 days) and then on a low calcium diet for 379 days. The bones show almost complete absence of trabeculæ of the bone shaft and a disappearance of the majority at the epiphyseal end (see Figs. 6 and 7).

Conclusions to Experiment 2.—The bony trabeculæ are diminished in an adult cat when kept on a low calcium diet and increased during a high calcium diet. No gross change in cortical thickness was demonstrable.

Experiment 3. The Location of New Calcium Deposits in Adult Cats Shown by Intravital Staining with Alizarin Red.

It has been adequately shown that alizarin red, when administered orally or intramuscularly will stain newly deposited bone (10–14).[5] This affords one an opportunity to show the exact location of newly formed bone.

[5] Since this paper went to press, we have discovered the experiments of John Hunter, done in 1794, in which he used madder in staining growing bone and teeth (The works of John Hunter, with notes, edited by James F. Palmer; Longman, Rees, Orme, Brown, Green, and Longman, London, 1837).

Twelve adult cats were first placed on the same low calcium diet used in Experiment 2. The group protocol is appended. Three died from snuffles early in the experiment. The remaining nine were kept on this low calcium diet for 82 days. It was thought that this was long enough to show a reduction in the number of bony trabeculæ. The cats were then placed on a high calcium diet and alizarin red was injected intramuscularly in order to stain the newly formed trabeculæ. After varying lengths of time on this treatment, the cats were killed. The bones were cleaned, scraped and cross-sectioned. The marrow contents were removed by digestion with pancreatic ferment at pH 8.3 for 24 to 36 hours. Cat 363 was killed on the 5th day and showed very few trabeculæ and these were stained very faint red (see Fig. 8). Cat 356 was sacrificed on the 10th day and as can be seen from Fig. 8, there were only a few trabeculæ but these were stained pink. Cats 362, 359, 360, 356, 357 and 361 were all sacrificed on the 55th day and as can be seen from Fig. 8, they all showed many red stained trabeculæ. Cat 358 was allowed to continue on the high calcium diet for 36 days longer without receiving the dye and showed not only red trabeculæ but also white trabeculæ. Presumably these white trabeculæ represent trabeculæ laid down during the period that the animal received no dye. The alizarin red deposited in all the bones was confined to the trabeculæ except for a small amount deposited around the vessels where they enter the endosteum.

Conclusions.—This group of experiments shows that the calcium stored while on a high calcium diet is deposited in the trabeculæ. The observation that alizarin red is deposited in new formed bone is confirmed.

Experiment 4. Control Experiments to Show that Alizarin Red Is Not Deposited in Bones during Periods of Calcium Equilibrium or Negative Calcium Balance.

Alizarin red is deposited in new formed bone but we have no conclusive proof that it is not deposited in previously formed trabeculæ. In order to investigate this, the following experiments were performed. (The group protocol is appended.)

1. Two cats (Nos. 383 and 384) had received a high calcium diet for months. This diet was continued and they were injected with alizarin red every other day for 21 days. If no new trabeculæ were formed during this period, their bones should show no stained trabeculæ, and the bones from both animals actually showed many trabeculæ but none were stained red (see Fig. 9).

2. Two cats (Nos. 355 and 365) had their left fore leg amputated at the shoulder joint under ether. They were then placed on a low calcium diet. After the 14th day they were injected with 40 units of parathormone daily. Starting

on the 20th day, they also received alizarin red every other day. On the 44th day they were both sacrificed. The photograph of the results (Fig. 6) demonstrates that the bones obtained after the low calcium diet plus parathormone régime have fewer trabeculæ—and none of these were stained red with alizarin.

3. Two cats (Nos. 380 and 381) which received a low calcium diet for about 8 weeks were continued on a low calcium diet and injected with alizarin red intramuscularly every other day. They were killed after they had been on alizarin for 20 days. Their bones showed a marked diminution in the number of trabeculæ and none of the trabeculæ were stained red (Fig. 9).

4. Two cats (Nos. 367 and 368) which had received a low calcium diet for 91 days had their left fore leg removed at the shoulder joint under ether and were then placed on a high calcium diet plus alizarin red. After they had been on this régime for 49 days they were sacrificed, and, as is seen in Fig. 8, the bones representing the low calcium diet showed few trabeculæ—whereas the ones representing the alizarin red—high calcium régime have an increased number of trabeculæ fairly well stained.

Conclusions.—Alizarin red, when administered to animals while in calcium equilibrium or negative calcium balance, does not stain the trabeculæ or previously formed bones. Therefore, we have further proof that alizarin red stains new formed bone.

Experiment 5. The Effect of Growth upon the Trabeculæ in Kittens.

Hamilton has demonstrated that normal infants must have a store of calcium at birth (aside from that contained in the milk ingested), which is sufficient to allow normal bone growth during the first 4 months of life. Could the trabeculæ possibly serve as this calcium supply which is called upon at this time for skeletal growth?

Experiment.—Five kittens were chosen for the experiment to note the effect of growth on the trabeculæ. The kittens were allowed to continue nursing. The first kitten killed at the age of 2 days showed the entire shaft to be filled with trabeculæ (see Fig. 10). The second kitten was killed at the age of 18 days and its bones showed a marked reduction in the number of trabeculæ when compared with those of the first kitten. The remaining kittens were killed on the 26th, 52nd and 67th days. As can be seen from Fig. 10, the older the kitten the fewer were the trabeculæ. This demonstrates that normal bone growth in young animals also results in a reduction of the readily available supply of calcium. Six normal growing kittens (not of the same litter) of varying ages were killed and their bones show very few spicules.

A normal kitten was allowed to continue nursing from its mother. Alizarin red ½ cc. was injected every day between the 20th and 30th days of life, and then

every 3 days up to the age of 60 days. The injections were then discontinued. The kitten was sacrificed at the age of 90 days. All the teeth became pink on the 30th day except for the very tips which remained white. Cross-section of the long bones showed the entire cortex to be stained red except near the epiphyseal ends where it was white. Again there were very few red stained spicules present showing that they were not being deposited.

This experiment is very important for comparison with the bones of adult cats on a high calcium diet while receiving alizarin red. The bones from the adult cats show no staining of the cortical bone except at the site of entry of the blood vessels, whereas the entire cortex of the kitten bones was red except for the area already mentioned. This white zone, in all probability, represented newly formed bone after the injections of dye had been stopped. This experiment not only proves that alizarin stains new formed bone but also adds further evidence to our thesis that very little calcium was laid down in the cortex of bones of our adult animals in Experiment 2.

Conclusions.—There is a marked reduction of the trabeculæ in kittens as a result of bone growth. Alizarin red when administered to a growing kitten is deposited in the cortex (shaft) of bones.

Experiment 6. Effect of Parathormone on the Trabeculæ in Growing Kittens and Rats.

Four kittens from the same litter were used, two for controls and two for parathormone injections. The two kittens receiving extract were started on parathormone at the age of 26 days. One control kitten died before the experiment was completed. During the next 56 days each extract kitten received 1270 units of parathormone or an average of 25.4 units per day. At the age of 82 days the three kittens were sacrificed. The serum calcium and phosphorus just before death (18 hours after the last injection) were as follows:

	Calcium *mg. per 100 cc.*	Phosphorus *mg. per 100 cc.*
No. 382, control	9.9	9.4
No. 383, extract	8.1	9.0
No. 384, extract	8.6	6.55

The bones from the extract animals showed no signs of decalcification by x-ray when compared with the normal control litter mate. There was no diminution in the number of trabeculæ present in the extract animals' bones when compared to the controls, but all three showed very few trabeculæ.

This same experiment was tried on two kittens from another litter. The extract was started on the 24th day and was continued for 121 days. During this time the kitten received 1090 units of parathormone. At the time of death the kitten receiving extract had a serum calcium of 10.7 and a serum phosphorus of 7.2. The control kitten had a serum calcium of 11.2 and phosphorus of 6.9. Again no decalcification or diminution of the bone trabeculæ could be demonstrated in the extract animal. The bones from both animals showed almost complete absence of the trabeculæ. Our failure to show signs of decalcification or diminution of trabeculæ in growing cats may be due to the fact that all the readily available calcium is used for normal bone growth.

All of the kittens which received parathormone showed no elevation of serum calcium; in fact it was slightly lower than in the control animals. We have had the same experience with adult cats. We administered as much as 100 units at a time, in eight such animals, and their blood, taken the 6th, 12th, 24th and 48th hours after injection, has never shown any elevation of the serum calcium.

Eight young growing rats from the same litter were all retained on a high calcium diet. At 10 days of age, four were started on parathormone, given intraperitoneally, and this was continued for 110 days. During this time they each received 885 units of parathormone. The serum calciums of the entire group at death were:

Control rats.	*mg. per 100 cc.*	Extract rats.	*mg. per 100 cc.*
No. 389	12.3	No. 389	13.9
No. 385	11.7	No. 385	13.0
No. 386	11.6	No. 386	11.75
No. 387	13.3	No. 390	12.8

X-ray examination of the bones of the animals which had received extract appeared to be much denser and shorter than their control siblings, and on cross-section they also showed many trabeculæ. We are unable to explain these findings.

Conclusions.—Parathormone administration to growing kittens does not result in any greater reduction of the trabeculæ than one observes in the course of normal bone growth. We have never been able to demonstrate a rise in serum calcium in cats or kittens after parathormone administration. Parathormone administration to growing rats results in a diminution in length of bone and an increase in the number of trabeculæ.

DISCUSSION.

The above studies prove that the trabeculæ of bones serve as a source of calcium. Here, as in many other respects, we have an

analogy between mineral and protein metabolism. It is the present conception that body protein is composed of structural protein and "deposit protein," and this latter decreases during protein starvation. So here the calcium of the bones is apparently partly structural (cortex) and partly a readily available reserve supply of calcium (trabeculæ). While large amounts of calcium are being used to form new structural elements as in growing animals, the trabeculæ are diminished. Hamilton (15) showed that in normal infants the ratio of calcium to body weight diminishes during the first 4 months. Inasmuch as the skeleton increases proportionately to the increase in weight, he concludes that a normal child has a store of calcium at birth to draw upon during the first 4 months. He further points out that this added store is deposited during the last months of pregnancy so that premature infants do not have this store. This, he believes, is why normal infants do not get rickets until 4 months of age and why premature infants almost invariably get rickets. These facts agree with those of Experiment 5 of our studies in which it was seen that kittens at birth have many trabeculæ which disappear as growth takes place. Autopsy material is now being collected to see whether the same is true of human infants.

A negative calcium balance decreases the trabeculæ and a positive balance increases them. The observation of Hunter and Aub (1) on the failure of parathormone to cause an increased excretion of lead during a second administration of the drug is now apparent. New trabeculæ, uncontaminated by lead, were laid down during the rest period between medication, and consequently a second administration of parathormone caused an elimination of extra calcium without an increased lead excretion. It does not seem unlikely that when the trabeculæ are depleted, the structural part of the skeleton meets demands for calcium, just as in the case of the structural proteins during starvation. But the bone shafts are extremely important in maintaining the body structure. Protected from depletion by this storehouse of readily available calcium, it is only in cases of great need that they are called upon to relinquish calcium. Thus, when the trabeculæ are not present to meet the demand for calcium to form new structural units during growth or stress, it is quite possible that calcium is drawn from the bone shafts, and rickets or osteomalacia

results. Such prolonged demand is not common but the appearance of osteomalacia after repeated pregnancies or Graves' disease is very suggestive evidence.

In summarizing the above experiments the following statements would seem justified.

Bone trabeculæ have as their function not only to act as a support against the tension of muscles (Carey) but to serve as the most readily available calcium supply in time of need. They can be influenced by many factors. Thus prolonged administration of parathormone in rabbits will decrease their number. Likewise a prolonged negative calcium balance will diminish these same bony spicules while a high calcium diet increases the amount of cancellous bone. Regardless of whether an animal is kept on a low or high calcium diet for a long period of time, no gross changes are noted in the cortex. The trabeculæ seemingly serve as the labile supply of calcium and the cortex as the stable supply. It would appear that anabolism and catabolism go on at a more or less constant rate in the cortical portion, except at times of unusual demand such as in osteomalacia.

Newly deposited calcium can be observed by intravital staining with alizarin red. By this method it has been established that when an adult animal is depositing calcium, newly formed trabeculæ are all stained red but the cortex remains unstained. This indicates that calcium was not being deposited in the cortex, or if so, it was at too slow a rate to be demonstrated by the deposition of the dye. When calcium equilibrium or a negative calcium balance is being maintained, the administration of alizarin red stains neither the cortex nor the trabeculæ. However, in growing kittens the administration of the same dye results in the entire cortex being stained red. This is further proof that the absence of the dye in the cortex of adult cats signifies that little or no calcium is being deposited in this portion.

The bones of kittens at birth are filled with trabeculæ. These rapidly disappear during the first weeks of life suggesting that they are needed for structural growth. Parathormone administration to cats is without effect; in them there is no resulting rise in the serum calcium and no demonstrable diminution in the number of trabeculæ. Yet its administration to growing rats results in an increased number of trabeculæ, a fact we do not know how to interpret.

CONCLUSIONS.

1. Bone trabeculæ are easily depleted by the prolonged administration of parathormone, long continued negative calcium balance and growth.

2. A long continued high calcium diet results in a rapid accumulation of the trabeculæ.

3. Alizarin red, as has previously been shown in the literature, is deposited in newly formed bone. Its use has made clear that easily mobilizable calcium is not deposited in the shafts of adult animals, but in the trabeculæ of bone.

4. The bone trabeculæ therefore serve as the storehouse of readily available calcium.

5. The shafts have a slow progressive exchange of inorganic salts and are not influenced except in the case of unusual body demands.

6. It is suggested that the absence of trabeculæ in premature infants and their depletion at the end of 4 months in a normal baby might well be an etiological factor in rickets.

7. In our observations, parathormone administration to growing or adult cats has been without effect.

8. Daily injections of parathormone in growing rats results in an increased number of trabeculæ and smaller bones.

Protocols for Experiment 2.

No. 337.—A brown and white, young female cat, weight 2.15 kilos, was placed on a low calcium diet on April 21, 1926. It remained on this diet until July 14, 1926 (84 days). It suffered from snuffles from May 21, 1926, to June 6, 1926. During this period the average daily food intake was: liver 102 gm., meat 99 gm., water 91 cc. The weight at this time was 3.5 kilos. On July 14, 1926, the left fore leg was amputated at the shoulder joint. Ether anesthesia was used. On July 20, 1926, the cat was placed on a known high calcium diet. The weight at this time was 3.0 kilos. The average daily intake for the ensuing 274 days was 440 cc. of milk. On April 15, 1927, the animal was sacrificed so that the bones from the one fore leg on a high calcium diet could be compared with the ones representing the low calcium diet period. At the end of the experiment the cat weighed 2.5 kilos. Autopsy showed no gross pathological changes.

No. 338.—A gray and brown, adult female cat weighing 2.95 kilos was placed on a low calcium diet April 21, 1926. On May 16, 1926, it gave birth to four dead kittens. On May 21, 1926, the cat contracted snuffles from which it recovered on June 9, 1926. It remained on this low calcium diet for 84 days.

During this time the average daily intake was: liver 96 gm., meat 76 gm. and water 80 cc. On July 13, 1926, the cat weighed 2.85 kilos. On this day the left fore leg was amputated at the shoulder joint under ether anesthesia. An uncomplicated postoperative period followed. On July 20, 1926, the weight was 2.45 kilos. The animal was then given a known high calcium diet and took an average of 206 cc. of milk per day for 150 days. At the end of this time it weighed 3.25 kilos. On December 22, 1926, the cat was sacrificed. Complete autopsy showed no gross abnormalities.

No. 339.—A white and gray, adult female cat, weight 3.25 kilos, was placed on a low calcium diet on April 21, 1926. On May 19, 1926, it developed snuffles. Under treatment it recovered on June 6, 1926. On June 16, 1926, the cat gave birth to six normal kittens. It was allowed to nurse these for a period of 2 weeks. The cat's average intake for 84 days was: liver 99 gm., meat 89 gm. and water 63 cc. At the end of this period it weighed 3.00 kilos. On July 14, 1926, the right fore leg was amputated at the shoulder joint under ether anesthesia. On July 20, 1926, the animal was started on a known high calcium diet. At this time it weighed 2.15 kilos. The average daily intake for the next 160 days was 358 cc. of milk per day. On December 29, 1926, the cat weighed 3.15 kilos. At this time it was sacrificed. No macroscopic pathological changes were noted at autopsy.

No. 341.—A gray and white, adult male cat weighing 3.95 kilos was placed on a low calcium diet on April 21, 1926. It consumed an average of 90 gm. of liver, 85 gm. of meat and 32 cc. of water per day for the next 84 days. At the end of this time it weighed 4.75 kilos. On July 14, 1926, the left fore leg was amputated at the shoulder joint under ether anesthesia. The animal was then placed on a milk diet which it took very poorly. On August 25, 1926, the weight had fallen to 2.95 kilos. At this point it was given raw eggs in addition to the milk. For the 267 days postoperative, it had an average daily intake of 78 cc. of milk and 2.43 eggs. At the end of this time the animal weighed 4.2 kilos. It was sacrificed on April 14, 1927.

No. 342.—A tiger-striped, adult male cat weighing 3.35 kilos was placed on a high calcium diet. On May 17, 1926, it contracted snuffles and did not recover until May 28, 1926. The average daily intake for 79 days was 286 cc. of milk. It then weighed 3.05 kilos. On July 13, 1926, the left fore leg was amputated at the shoulder joint under ether anesthesia. 7 days later the animal was placed on a low calcium diet. For 269 days the average daily intake was: 79 gm. of meat, 99 gm. of liver and 50 cc. of water. At this time it weighed 4.2 kilos. On April 14, 1927, it was sacrificed. The autopsy was negative.

No. 343.—A white, tiger-striped adult male cat, weighing 3.0 kilos, was started on a high calcium diet on April 24, 1926. On May 23, 1926, it showed signs of snuffles. These disappeared after May 30, 1926. The cat consumed an average of 508 cc. of milk during the next 79 days and weighed 3.40 kilos. On July 13, 1926, the left fore leg was removed at the shoulder joint under ether anesthesia.

On July 20, 1926, the animal was started on a low calcium diet and kept on it for 267 days. The average daily intake for this period was: meat 60 gm., liver 100 gm. and water 50 cc. At the end of this period it weighed 3.75 kilos. On April 13, 1927, it was sacrificed. Autopsy showed no gross pathology.

No. 344.—A brown and white, adult male cat, weighing 2.85 kilos, was placed on a high calcium diet on April 25, 1926. It had signs of snuffles on May 23, 1926. The nose became cold and all signs of snuffles disappeared on June 6, 1926. The average daily intake for a period of 80 days was 324 cc. of milk. The weight at this time was 2.45 kilos. On July 13, 1926, the left fore leg was amputated at the shoulder joint under ether anesthesia. On July 20, 1926, the cat was placed on a low calcium diet. It remained on this diet in excellent physical condition until it was sacrificed August 2, 1927, a period of 379 days. During this time the average daily intake of food was: liver 100 gm., meat 50 gm. and water 52 cc.

No. 345.—An all black, adult male cat, weighing 2.80 kilos, was put on a high calcium diet on April 25, 1926. The average intake for the next 80 days was 318 cc. of milk. At the end of this time it weighed 3.1 kilos. On July 13, 1926, the left fore leg was amputated at the shoulder joint under ether anesthesia. On July 20, 1926, the cat was placed on a low calcium diet. The average daily intake for 161 days was: liver 100 gm., meat 62 gm. and water 80 cc. At the end of this period it weighed 3.90 kilos. On December 27, 1926, the animal was sacrificed. Autopsy showed no gross pathological changes.

No. 346.—An adult, black and white female cat weighing 2.95 kilos was placed on a high calcium diet on April 25, 1926. On May 22, 1926, the cat developed snuffles. All signs of snuffles were absent on May 30, 1926. On June 22, 1926, three fat, normal appearing kittens were born and were not weaned until they were 2 weeks of age. During this 79 day period the cat had an average daily intake of 296 cc. of milk. The weight at this time was 2.70 kilos. On August 14, 1926, the left fore leg was amputated at the shoulder joint under ether anesthesia, 6 days later the cat was placed on a low calcium diet. For a period of 157 days, the cat had an average daily intake of: liver 98 gm., meat 48 gm. and water 28 cc. On December 22, 1926, the cat was sacrificed. At this time it weighed 3.55 kilos. The autopsy findings were negative.

Protocols for Experiment 3.

On January 18, 1927, twelve cats were placed on the same low calcium diet. On this régime they all received approximately 13 mg. of calcium per day. Three cats (Nos. 355, 365 and 366) contracted snuffles on January 28, 1927, were isolated and later killed. The nine remaining cats were kept on the low calcium diet for 82 days. On April 10,1927, they were all placed on a high calcium diet. They also received 2 cc. of alizarin red intramuscularly the next 4 days. On the 5th day Cat 363 was killed and the bones were prepared and saved for comparison. They were then given 1 cc. of alizarin intramuscularly every day. On June 3, 1927, Cats 357, 358, 359, 361 and 364 were found to have fluctuating areas at the

site of injection of the alizarin red. They were all incised under ether and red stained material was obtained, no frank pus. Starting on April 18, 1927, each cat received 1 cc. of alizarin every other day. On April 30, Cat 356 was killed and the bones were prepared and saved for comparison.

From this date they received 1 cc. of alizarin every 3 days. On June 3, the following cats were killed: Nos. 356, 357, 360, 361, 362 and 364. The bones were all prepared in the same manner. Cat 358 was given no more injections but was kept on a high calcium diet. It was sacrificed on July 9, 1927. The bones were prepared in the same manner. None of the cats showed any gross pathological changes at autopsy.

Protocols for Experiment 4.

Two normal cats (Nos. 380 and 381) which had been on an unlimited high calcium diet for weeks were placed on a known high calcium diet on June 13, 1927. On July 11, 1927, they were started on alizarin red ½ cc. every other day. On July 31, 1927, they were both sacrificed. They had both gained weight during the period of observation. No gross pathological changes were noted at autopsy.

Two cats (Nos. 355 and 365) that had previously been on an unlimited high calcium diet had their left fore leg amputated at the shoulder joint on June 15, 1927, under ether anesthesia. On June 16, No. 355 weighed 2.25 kilos and No. 365 weighed 2.70 kilos. On this same day they were placed on a low calcium diet. From July 2 on they were both given 40 units of parathormone per day. On July 7 they were also started on alizarin red ½ cc. every other day. They were sacrificed on July 30 for comparison of the bones and to see if the spicules had been stained by the dye. At the time of death, No. 355 weighed 2.45 kilos and No. 365 weighed 3.1 kilos. There were no gross pathological findings at autopsy.

Two cats (Nos. 383 and 384) which had been on a low calcium diet for 49 days were placed in cages on June 13, 1927, and the same diet continued. Starting on July 11, 1927, they were given ½ cc. of alizarin red every other day. On July 31, 1927, they were sacrificed to see if the spicules were stained red. Their weights had remained stationary. The autopsies were negative.

Two cats (Nos. 367 and 368) which had been on a low calcium diet for 91 days had their left fore leg amputated at the shoulder joint under ether anesthesia. On June 15, 1927, No. 367 weighed 3.25 kilos and on June 16, No. 368 weighed 2.15 kilos. On June 30 they were placed on a high calcium diet and alizarin red. They were both sacrificed on August 18, 1927, for comparison of the bones. Their weights at this time were: No. 367—3.45 kilos; No. 368—2.60 kilos. The autopsies were negative.

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EXPLANATION OF PLATES.

PLATE 13.

FIG. 1. Shows the effect of long continued administration of parathormone upon the bones of rabbits. The bones of Rabbit 301 (extract) show marked signs of decalcification as compared to its control litter mate, No. 314.

PLATE 14.

FIG. 2. The effect of parathormone on the cancellous bones. The femurs of Rabbits 301, 298 and 327 show fewer trabeculæ than do the femurs of their control litter mates, Nos. 297 and 314.

FIG. 3. The effect of diet on the amount of cancellous bone. Cat 342. The upper humerus represents the high calcium diet period and contains many more trabeculæ than its opposite member (the lower humerus) representing the low calcium diet period.

FIG. 4. The effect of diet on the amount of cancellous bone. Cat 339. The upper humerus represents the high calcium diet period and contains many more trabeculæ than its opposite member (the lower humerus) representing the low calcium diet period.

PLATE 15.

FIG. 5. A photograph showing effect of diet upon the amount of cancellous bone. The humerus representing the high calcium diet period is placed above its opposite member representing the low calcium diet period. The bones are arranged in rows according to the dietary régime and each pair is from a different animal. The epiphysis is missing from the humerus representing the high calcium diet period of Cat 343, and also from the humerus representing the low calcium diet of Cat 345.

FIG. 6. The effect of diet and parathormone upon the amount of cancellous bone. The humerus representing the high calcium diet period is placed in the upper row and its opposite member representing a low calcium diet is placed in the lower row. The changes are most marked in Cat 344, which did not receive parathormone. (Refer also to Experiment 4 in text.)

FIG. 7. The effect of diet on the trabeculæ. The upper femur (Cat 344) shows the absence of trabeculæ after a long period on a low calcium diet, whereas the femur from Cat 339 shows many trabeculæ as a result of a period on a high calcium diet.

PLATE 16.

FIG. 8. Showing the location of new calcium deposits in adult cats by the injection of alizarin red while the animals were on a high calcium diet. The bones representing Cats 356, 357, 358, 359, 360, 361, 362, 363 and 364, have been fully described under Experiment 3 (see text). The bones from Cats 367 and 368 are so arranged that the bone above the number represents the low calcium diet period and that below represents the high calcium diet plus alizarin red period. (For further details, see Experiment 4, Cats 367 and 368.)

PLATE 17.

FIG. 9. The effect of diet on the trabeculæ. The bones from Cats 367, 383 and 384 show many trabeculæ after being on a high calcium diet. Cats 368, 380 and 381 show a diminution in the number of trabeculæ as a result of long continued negative calcium balance.

FIG. 10. The effect of growth on the trabeculæ. The bones of the kittens described in Experiment 5 are shown here arranged in the order of age. They show a diminution in the number of trabeculæ as the kittens grow older.

FIG. 1.

(Bauer, Aub, and Albright: Calcium and phosphorus metabolism. V.)

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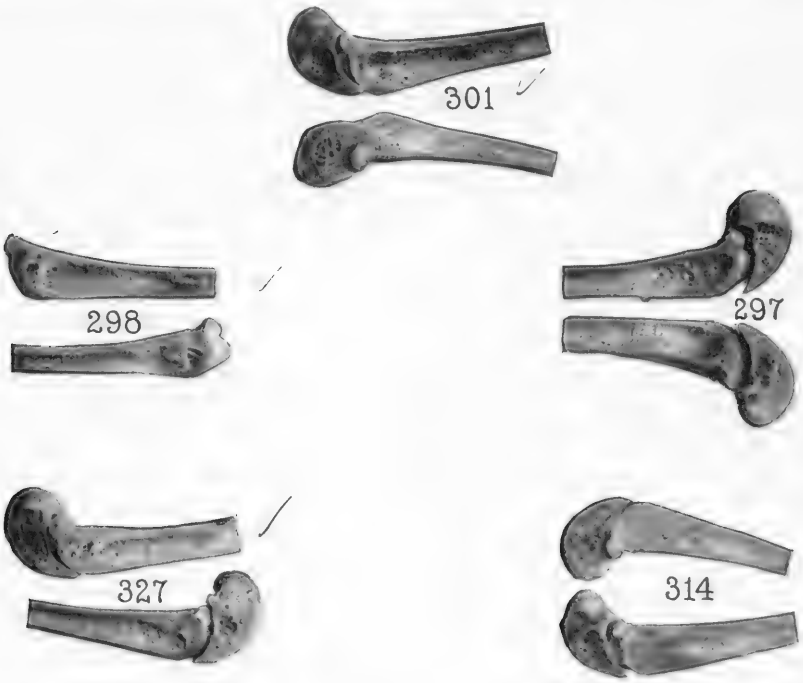
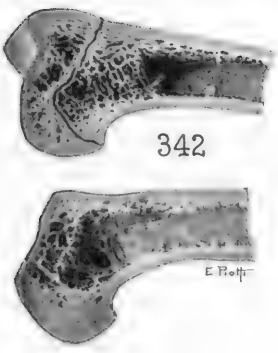


FIG. 2.



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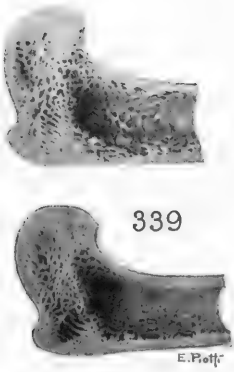
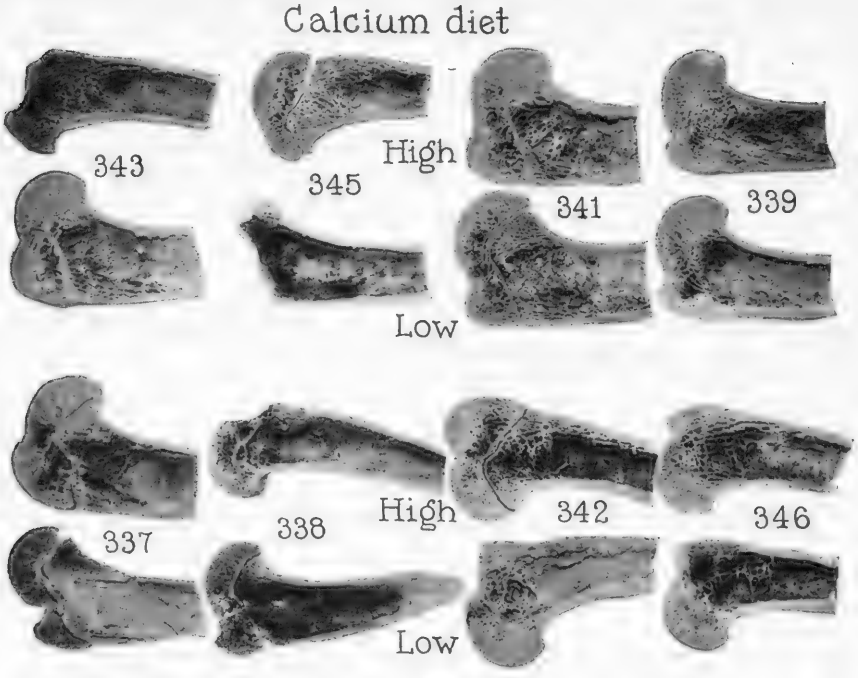


FIG. 4.

(Bauer, Aub, and Albright: Calcium and phosphorus metabolism. V.)



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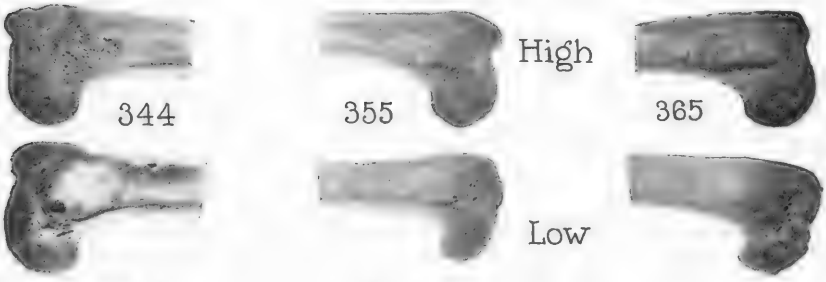

FIG. 6.

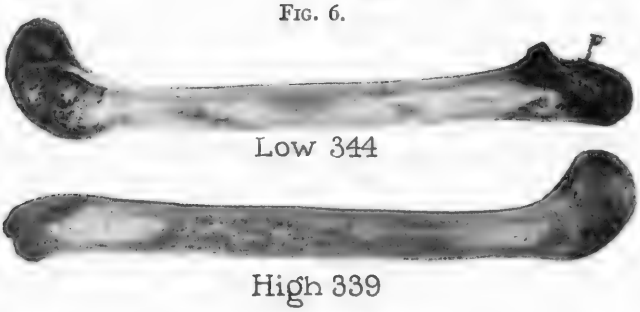

FIG. 7.

(Bauer, Aub, and Albright: Calcium and phosphorus metabolism. V.)

FIG. 8.

(Bauer, Aub, and Albright: Calcium and phosphorus metabolism. V.)

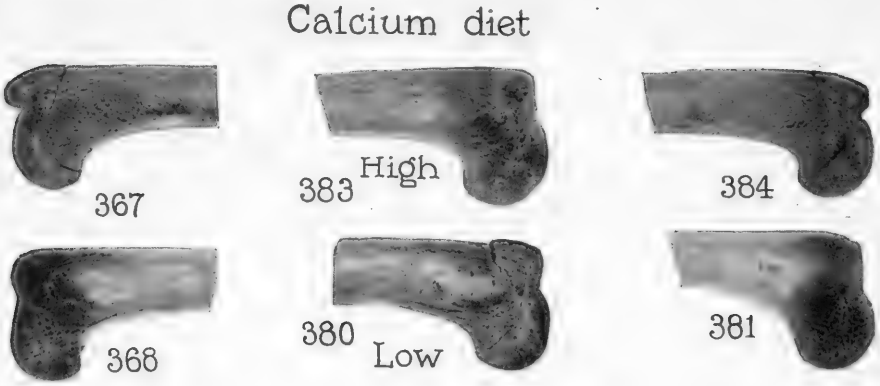

FIG. 9.

FIG. 10.

(Bauer, Aub, and Albright: Calcium and phosphorus metabolism. V.)

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AUGUST 1, 1928.

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